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CD90 as a Target for In Vivo Gene and Cell Therapies

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Abstract

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Hematopoiesis occurs through a hierarchical process of differentiation beginning with multipotent self-renewing hematopoietic stem cells (HSCs) that persist throughout the human lifespan and ending with transient terminally differentiated unipotent cells. This progression allows for a malleable hematopoietic system capable of responding to pathological and physical assaults to the body. The hierarchical differentiation of the hematopoietic system can be exploited in the clinic for benign and malignant hematology. A patient's pathological hematopoietic system can be cured by replacing their HSCs with new HSCs from a healthy donor (i.e. allogeneic transplantation). Furthermore, the advent of gene modification therapies allows for pathological mutations in a patient's HSCs to be treated by gene therapy and re-transfused into patients (i.e. autologous transplantation). The best examples of this therapeutic platform being the treatment of hemoglobinopathies.

The reliance of current ex vivo HSC gene therapy approaches on prolonged *in vitro* culture time, toxic myeloablative conditioning regimens to remove unmodified HSCs, and highly specialized infrastructure severely impact the accessibility of these therapies. Therefore, novel HSC-specific gene therapy platforms with simplified manufacturing protocols for *in vivo* applications are needed. Previous work refined the target for HSC gene therapy and identified a phenotypically defined subset of cells (CD34⁺CD90⁺) that is exclusively responsible for rapid

recovery onset, robust long-term multilineage engraftment, and entire reconstitution of the bone marrow (BM) stem cell compartment. My long-term goal is to target this refined HSC subset *ex vivo* and *in vivo* for gene and cellular therapies. In this dissertation I develop CD90-recognizing chimeric molecules successfully engineered them onto viral vectors. First proof-of-concept studies *in vitro* showed that CD90-targeted lentiviral vectors (CD90-LVs) delivered their cargo, with high specificity, into CD90⁺ cell lines and primary human CD34⁺CD90⁺ HSCs. Encouraged by these results, we applied and evaluated the safety of CD90-targeted viral vectors for *in vivo* applications to transduce and edit human HSCs.

Sufficient conditioning with low-dose chemotherapeutics before transplantation of gene-edited HSC is crucial for efficient *ex vivo* HSC gene therapy. This pretreatment, while genotoxic, enables the engraftment of gene-therapy cell products at therapeutic chimeric thresholds. *In vivo* gene therapy does not require pre-conditioning. Therefore, targeted, nongenotoxic selection platforms post-vector delivery can be leveraged to increase gene marked/corrected chimerism. Targeting the CD34⁺CD90⁺ HSC pool for these enrichment strategies would significantly improve gene modified chimerism. I hypothesized that using CD90-targeted gene and cellular therapies *in vivo* will not only efficiently deliver gene therapeutics to human HSCs with high target specificity, but gene-marked/edited HSCs can subsequently repopulate the hematopoietic hierarchy after anti-CD90-targeted selection (i.e., chimeric antigen receptor (CAR) T cells).

In summary, CD90-LVs can be easily implemented into currently existing *ex vivo* HSC gene therapy approaches to replace the utilization of cytotoxic chemical transduction enhancers and increase the efficiency of HSC delivery. Furthermore, the ability to target LVs to HSCs *in vivo* will significantly enhance the accessibility of HSC gene therapy in areas with limited biomedical infrastructure. Additionally, protecting HSCs from CD90-targeted immunotherapies and discovering CD90-dependent signaling pathways will minimize off-target toxicities of potential

therapies and open a new category of anti-tumor targets in the clinic. This strategy could also enrich gene-edited HSCs to treat sickle cell disease (SCD).

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Data and Code Availability

Demultiplexed sequence datasets for all samples included in this work are available on NCBI's Sequence Read Archive through BioProject accession PRJNA1199447. Any additional information is available upon request.

Introduction

In 1958, Dr. E. Donnell Thomas performed the first successful bone marrow transplantation (BMT) in a patient with aplastic anemia who received marrow from an identical twin [1]. This began the genesis and revolution of cell therapeutics for the treatment of hematologic disorders. Dr. Thomas's diligent pursuit of deriving a refined BMT protocol, as well as his contemporaries' landmark discoveries of HLA typing and conditioning regimens, has now enabled the treatment of thousands of patients with nonmalignant and malignant hematologic disorders. The biological basis of why BMT works stems from the pioneering work of Dr. James Till and Ernest McCulloh, who hypothesized and experimentally validated the presence of a singular cell capable of engrafting and repopulating the bone marrow niche after transplantation, while also maintaining its self-renewal capacity [2]. This powerful principle that a singular cell could be responsible for the derivation of an entire immune system, as well as the hemostasis physiology of a mammal had broad implications for clinical practice if it could be isolated, molecularly manipulated, and transplanted (i.e gene therapy).

It was the work of Dr. Irving Weissmann and Dr. John E. Dick to isolate and molecularly define the immunophenotype of this potent hematopoietic stem cell through laborious murine transplantation experiments [3-6]. In a landmark study published in 1988 in the Science Journal, Dr. Weissmann's group defined and isolated the human HSC as a lineage negative (lin-), CD34+CD90+ population [5]. Follow-up studies from the Weissmann group further molecularly defined the HSC population's immunophenotype as being lin-CD45+CD34+CD45RA-CD38-CD90+ [3]. This molecular definition enabled the isolation of a potent population, in which one in every 100 cells could fulfill the HSC functional definition. John

Dick's group further defined the molecular immunophenotype of the HSC population by discovering the presence of CD49f, which can isolate a more potent and homogeneous subset of Dr. Weissman's isolation schema [4]. The addition of using CD49f allowed for the isolation of a population in which one in every three cells could fulfill the HSC functional definition.

As new protocols for HSC isolation were being developed, new tools for manipulating the genome of HSCs were being evaluated preclinically. Dr. Luigi Naldini's invention of the lentivirus system enabled the transfer of gene sequences into the genomes of dividing and nondividing cells [7]. This began the advent of the gene therapy era. Even still, new tools have been developed not only to insert new genes into the genome, but also to alter the sequence of the endogenous genome (e.g., CRISPR, TALEN, Base Editing, Prime Editing) [8]. This expansive toolbox enables the treatment of most genetically derived hematological pathologies, including Sickle Cell Disease, Fanconi Anemia, and many others. Hematopoietic stem cell (HSC) gene therapy is, therefore, the genetic engineering — either by gene transfer or altering the endogenous genetic code (i.e., gene editing) — of HSCs, followed by the subsequent transplant of manipulated HSCs. Today, HSC gene therapy is not limited by the lack of capacity to isolate, transplant, or genetically manipulate HSCs but rather the cost and laborious process of performing the protocol.

The Kiem group has endeavored to streamline the protocol of HSC gene therapy, reduce the consumable cost of the protocol, and increase its efficiency [9-11]. Clonal tracking of nonhuman primate (NHP) hematopoietic stem and progenitor cell (HSPC) population subsets that underwent lentiviral gene transfer revealed that the CD34+CD90+ subset of HSPCs was responsible for both short-term and long-term reconstitution of the BM niche as such Dr. Kiem and colleagues argued that HSC gene therapy only required the gene modification of this subset for successful HSC gene therapy [9]. Reducing the pool of cells that need to be genetically modified significantly reduces the cost of consumables as well as ensures long-term persistence of the gene therapy. Even still, while the CD90-targeted protocol is a significant

advancement in streamlining HSC gene therapy, it does not have a broad reach to developing countries outside of the United States, Europe, and China. The goal of my dissertation was to bridge this gap and further democratize HSC gene therapy by developing an in vivo HSC gene therapy platform that targets the CD34⁺CD90⁺ pool using viral particles. Furthermore, this dissertation aimed to understand the biology of CD90 further, in the hope of developing stem cell-specific therapeutics.

The chapters are developed through a logical flow of how we created these novel CD90-targeted therapeutics. It begins with a proof-of-principle study of developing the first CD90-targeted viral particles. These findings helped establish that such a strategy was feasible and could indeed target HSCs. Furthermore, it provides the basis for quality control of such a novel platform. Following the proof-of-principle study, Chapter 2 validates the capacity of CD90-targeted viral particles to gene-modify HSCs in vivo without perturbing either self-renewal capacity or differentiation. Chapter 3 builds on the principle of targeting HSCs in vivo by developing a strategy to program HSCs to proliferate, thus increasing the abundance of gene-modified HSCs in vivo. Finally, Chapter 4 answers the question of whether CD90 plays a crucial role in adult HSC biology.

Chapter 1. Proof of Principle Targeting of CD90⁺ HSCs with VSVG pseudotyped Lentivirus

1.1 Introduction

Previous work in the lab has shown that the CD90⁺ subset of CD34⁺ HSPCs is exclusively responsible for rapid recovery onset, robust long-term multilineage engraftment, and complete reconstitution of the bone marrow (BM) stem cell compartment [9-11]. Furthermore, CD34⁺CD90⁺ cells are almost entirely depleted of phenotypically, transcriptionally, and functionally committed progenitor cells [12]. Isolation and direct targeting of this HSC-enriched subset from mobilized human apheresis products *ex vivo* significantly increased

lentivirus-mediated gene transfer without impacting the cells' long-term multilineage engraftment potential.

To enable highly specific targeting of CD90⁺ HSCs, we designed CD90-targeted viral vectors engineering an anti-CD90 single chain variable fragment (scFv) onto VSV-G-pseudo typed lentiviral vectors. CD90-targeted viral vectors were further knocked out for their native targeting to optimize on-target specificity. Lastly, we developed a hydrodynamics-based methodology to easily and reliably titrate and validate our surface-engineered viral vectors.

1.2.1 VSV-G-Based Viral Vectors Can Be Targeted to CD90

In addition to measles-pseudotyped viral vectors, we next evaluated the VSV-G-based system for surface engineering following the previously described strategy by Dobson et al.[13] Native targeting of the VSV-G envelope for the LDL-receptor (LDL-R) were further knocked out to introduce specific point mutations as described by Nikolic et al [14]. The CD90 scFv was introduced onto the capsid using a CD8 hinge and ICAM domain. Similar to the measles vectors, four VSV-G variants were generated with V1 (WT) recognizing LDL-R, V2 (KO) removing LDLR recognition, V3 (WT/CD90) recognizing LDL-R and CD90, and V4 (KO/CD90) only binding to CD90 (**Figure 1A**).

The number of total viral as well as RNA-loaded particles was determined using the ZetaView® instrument (**Figure 1B**). Regardless of the modifications and addition of the CD90 scFv, all four envelope variants equally produced viral particles. However, the loading efficiency of viral particles (frequency of RNA-loaded particles within total particles) decreased as more engineering was done with the lowest loading efficiency seen in V4.

To test functionality of the surface-engineered VSV-G vectors, K562 cells were exposed to increasing doses of all four viruses taking the number of RNA filled capsids into account determined by NTA (**Figure 1C**). Similar to the measles variants, the V2 (KO) variant failed to

fuse with the cells, the V3 (WT/CD90) version demonstrated a gain of function relative to V1 (WT), and V4 (KO/CD90) regained the ability to fuse with cells comparable to V1 (WT). Transduction efficiency of K562 for variants V1, V3, and V4 was dose-dependent in a linear fashion until reaching a plateau at about 50% already at low particle number for the V3 variant, with no unwanted uptake seen for the KO variant V2 even in the highest dose.

1.2.2 On-Target Efficiency of CD90-Targeted Viral Vectors

To test the on- and off-target activity of our CD90-targeted measles- as well as VSV-G-pseudotyped viral vectors, we performed co-culture experiments mixing CD90-lacking (off-target) and CD90-expressing (on-target) suspension cell lines. Raji cells, human B lymphoblastoid cells, do present very low levels of CD90 protein on the cell surface (off-target), whereas Jurkat cells, immortalized human T lymphocytes, express CD90 protein at high levels (on-target) (**Figure 2A**). GFP-transduced Jurkat cells and GFP⁻ Raji cells were mixed and exposed to measles and VSV-G variants 1 and 4 in a serum-free, transduction enhancer-free, suspension culture system, and mScarlet expression flow-cytometrically determined on day 3-5 post-transduction (**Figure 2B**). When the variant H1/V1 (WT) virus was used, mScarlet expression was seen for Raji as well as Jurkat cells by flowcytometry. However, when the cell mix was exposed to variant H4/V4 (KO/CD90), a strong preference of mScarlet signal was seen in the CD90⁺ Jurkat cells (on-target) and only minimal transduction in the CD90^{low/-} Raji cells (off-target) (**Figure 2B**). To quantify the on-target specificity, we calculated the ratio of mScarlet⁺GFP⁻ cells within total GFP⁻ Raji to mScarlet⁺GFP⁺ cells within total GFP⁺ Jurkat cells (**Figure 2C**). For both virus types, the CD90-targeted variant 4 showed significantly higher on-target specificity in comparison to the variant 1 (WT) confirmed CD90-mediated target specificity. All targeting experiments were performed without the addition of any transduction enhancer such as protamine sulfate as such additives led to expected improvement of transduction for WT virus (here Variant H1) but an unspecific fusion of our KO variants (here

Variant H2) in a dose-dependent manner (**Figure 2D**). Finally, VSVG variants 1 and 4 were applied to human GCSF-mobilized CD34⁺ hematopoietic stem and progenitor cells to evaluate the targeting specificity. Similar to experiments on cell lines, our CD90-targeted variant 4 demonstrated significantly greater transduction of the CD34⁺CD90⁺ cell fraction in comparison to the WT variant V1 (**Figure 2E**). On-target specificity of this CD90-targeted virus was entirely abrogated when the transduction enhancer protamine sulfate was used validating that CD90-specificity of our virus is mediated by the recognition of the antigen through the added scFv.

1.3. DISCUSSION

Here we demonstrate the successful design of measles and VSV-G pseudotyped lentiviral vectors with a novel anti-CD90 scFV to target HSCs. Surface engineering did neither impact binding nor fusion of viral vectors to CD90-expressing cell lines and primary human HSCs. Most importantly, targeted vectors demonstrated enhanced on-target specificity in mixed cultures of cell lines as well as for human HSCs within bulk CD34⁺ HSPCs *ex vivo*. We further describe novel NTA-based methods to reliably validate surface engineered viral vectors measuring their size, loading-efficiency, and targeting functionality. Hydrodynamic titration of engineered vectors is highly reproducible and predictive of the transduction efficiency, providing a reliable surrogate to functional readouts on cell lines which are not applicable to surface engineered vectors.

The development of targeted gene therapy agents has been very successful for non-hematological diseases.[15-19] Previous work has shown that by conjugating antibodies or scFvs to the surface of nanoparticles (NPs) and viruses, one can reliably target cells of interest.[15, 18, 20-28] Targeted delivery, however, depends on the uptake of the vector after attaching to the cell/antigen, which could not be achieved with CD34-targeted HSC gene

therapy vectors.[20] While this previous study aimed to integrate the anti-CD34 scFv between the fusion protein and signaling peptide of the murine leukemia virus (MuLV) envelope protein, similar to our Measles-based modification of the hemagglutinin, we here used a more recent strategy reported by Funke et al.[29] , independently co-expressing the anti-CD90 scFv together with either a wild-type or mutated version of the fusion protein on the VSV-G envelope. Adapting this strategy for VSV-based envelopes, we successfully designed CD90-targeted viral vectors that fused and transduced CD90-expressing cell lines as well as CD34⁺CD90⁺ HSCs with secondary colony-forming potential. Although the exact mechanism of CD90-mediated cellular uptake is not known, cytomegalovirus (CMV) and Human Immunodeficiency Virus (HIV) use CD90 as a crucial part of their cellular entry process.[30-32] CMV entry was interrupted when CD90 was either blocked with an antibody or downregulated using an siRNA approach.[32] In addition, liposomes conjugated with anti-CD90 antibodies have been shown to be capable of binding and internalizing through the CD90 antigen on liver cancer cells, demonstrating efficient uptake of CD90-targeted lipid nanoparticles (LNPs) also *in vivo*. [33] Our viral vectors have been genetically modified to lose their native binding (LDL-R for VSV-G; CD46 and CD150 for measles), but their ability to fuse with the target cell remains intact. Thus, the CD90 scFv enables cell-specific binding while viral entry remains dependent on the viral envelope proteins. More in-depth studies will be required to determine whether CD90 is actively involved in the uptake, which pathways are triggered, and whether this system can also be used completely independently from viral fusion proteins. Regardless of the exact pathways involved, the CD90 protein was targeted to primary human HSCs without any obvious impact on the cells' proliferation and differentiation potential. Follow-up work will be needed to evaluate the recycling and re-expression of CD90 protein on the cell surface after CD90-mediated viral entry to ensure that the long-term multilineage engraftment of HSCs is warranted.

A major bottleneck for the development of surface engineered viral vectors for HSC gene therapy is the inability to quickly and reliably verify the vectors before use. Genetically

engineered viral vectors lose their native binding and functional titration methods on HEK293T or HT1080 cell lines are not possible. While the lack of cellular uptake can be overcome using qPCR-based technologies to quantify RNA-loaded viral particles, functional qualification of the surface-modification cannot be achieved with genomics. Validation of antigen/target recognition is needed to ensure that surface-engineered viral vectors are functional and targeting ligands have been successfully integrated into the capsid and correctly folded. While protein-protein interaction is traditionally demonstrated using SEC or biolayer interferometry, heterogeneity of viral particles and contamination with other proteins during vector preparation do not allow reliable testing using common technologies. To close this gap, we here developed a novel methodology to titrate and validate viral vectors using nanoparticle tracking analysis (NTA).

NTA is commonly used to measure the size, charge, concentration, and heterogeneity/polydispersity of nanoparticles such as LNPs and PNPs, as well as exosomes[34-36] isolated from human specimens and cell culture supernatant. Here we show that NTA also allows for the efficient and reliable quantification of viral vector concentration, size, and RNA loading. Most importantly, hydrodynamic viral titers correlated with the transduction efficiency on cell lines for wild-type variants, providing a surrogate method for the traditional titration on cell lines or other qPCR-based strategies. NTA was further used to demonstrate successful antigen recognition of targeted vectors. Custom-designed fluorochrome-conjugated CD90 protein was bound by the scFv-decorated VSV-G- and measles-pseudotyped viral vectors, resulting in a size increase measured by NTA and confirming fully functional antigen recognition when the targeting ligand was presented on the virus, similar to SEC, demonstrating the interaction of proteins resulting in a size increase and enhanced retention time. The measurement of these parameters is nearly instantaneous and provides a new alternative to traditional titration methods of wild-type, novel surface-engineered viral vectors, as well as virus-like particles. Viral titers can even be evaluated from the supernatant before purification and concentration to combat the observed technical variability in

the number of total particles and mRNA loading. The assessment of supernatants will allow the discontinuation of suboptimal preparations and high-throughput optimization of conditions to gain greater yields and better loading efficiencies. As viral vectors often undergo freezing and thawing, quality control and quantification of viral particles can even be performed right before applying them, providing an opportunity to adjust and carefully control viral titers in real-time, removing a factor of variability in virus-mediated cell therapy applications.

Surface engineering of viral capsid bears the risk of decreased titers and RNA loading efficiency as previously reported by Friedel. et al.[37] While no to minimal impact was seen for CD90-targeted Measles-pseudotyped viral vectors, we observed a drop in the number of RNA-loaded viral particles for VSVG-based variants adding the anti-CD90 scFv on the capsid and/or introducing the mutations to abolish LDL-R recognition. Although the impact of surface engineering on VSVG-based vectors was mild, further evolution of the scFv and removing potentially problematic amino acid residues that can either interfere with folding of the targeting ligand, impact the integrity of the capsid, or lower the RNA-loading efficiency will be needed. Alternatively, other viral capsids in the VSV family such as Cocal[38-40] should be evaluated for surface engineering and consequently the impact on viral titers.

A major advantage of our CD90-targeted viral vectors is the ability to incorporate them into already existing *ex vivo* gene therapy pipelines focusing on the gene modification of CD34⁺ HSPCs. Further purification of the CD90⁺ subset for direct targeting and to enhance the transduction efficiency [10] would potentially no longer be needed to transduce long-term engrafting HSCs and likely require even lower viral titers, reducing the overall costs. Even the purification of CD34⁺ cells from the leukapheresis products may no longer be necessary, providing a novel vector to realize closed-system vein-to-vein applications with the transduction of cells in bags and immediate infusion of the transduced cells back into the patient. Finally, direct intravenous or intraosseous injection of the HSC-targeted viral vectors would be warranted to further simplify HSC gene therapy, providing full portability and, consequently,

better accessibility. However, before such targeted vectors can enter the clinical routine, comprehensive *ex vivo* and *in vivo* studies in humanized mice as well as large animals such as the nonhuman primate will be needed to test the long-term impact of CD90-mediated transduction, confirm on-target specificity, determine the half-life/pharmacokinetics in circulation, evaluate serum susceptibility, and analyze potential off-target effects.

Especially for *in vivo* applications, various avenues need to be explored in order to provide the best targeting, highest efficiency, and least off-target effects. Adaptation of the targeting system using Cocal-pseudotyped lentiviral vectors, which are less susceptible to serum inactivation, may be favorable for *in vivo* administrations.[38, 39] Furthermore, the targeting of multiple HSC-associated cell surface markers using colocalization-dependent protein switches (Co-LOCKR) protein switches may help to increase the on-target specificity.[41] Finally, adaptation of our targeting ligand for use with non-viral delivery platforms such as polymer or lipid nanoparticles,[42] as well as virus like particles,[43] may enable the transient delivery of nucleases or therapeutic mRNAs and proteins without the risk of insertional mutagenesis.

Our new CD90-targeted vectors have the potential to improve already existing *ex vivo* HSC gene therapies as well as overcome current hurdles to apply gene therapy *in vivo*. The development of targeted viral vectors has the potential to increase the on-target efficiency, safety, and accessibility of HSC gene transfer for a variety of different diseases and will be a crucial step in democratizing access to gene therapies.

2.4 Figures and Legends

Figure 1

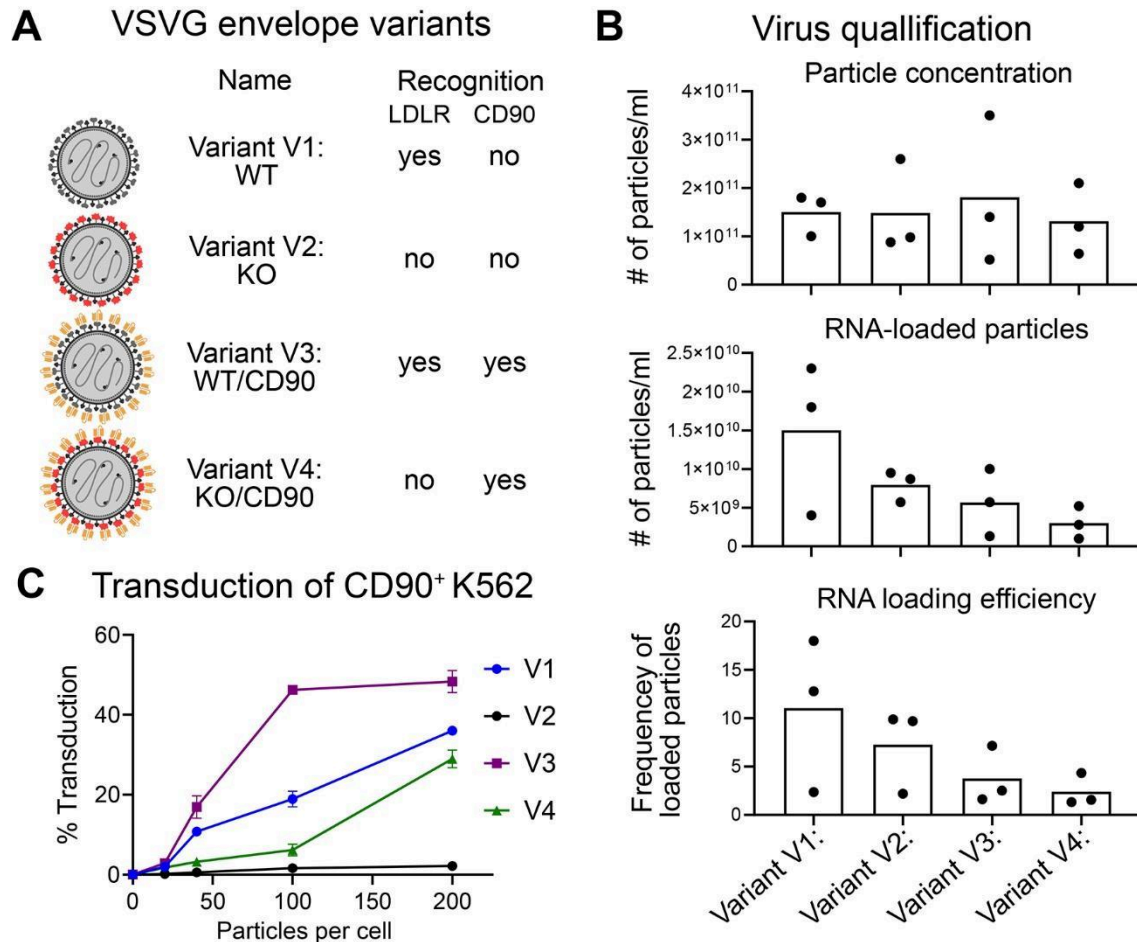


Figure 1: Design and quality control of CD90-targeted VSV-G-pseudotyped lentiviral vectors. (A) Summary of designed VSV-G envelopes and the expected antigen recognition. (B) Concentration of total and RNA-loaded viral particles per ml culture medium measured with NTA (top two graphs). Calculated RNA loading efficiency (bottom graph). (C) Transduction of 200.000 K562 cells with all four VSV-G variants at different doses as indicated below the graph. Transduction efficiency (mScarlet expression) measured by flow-cytometry. (Statistics: Mean±SD, T-test, significance as indicated).

Figure 2

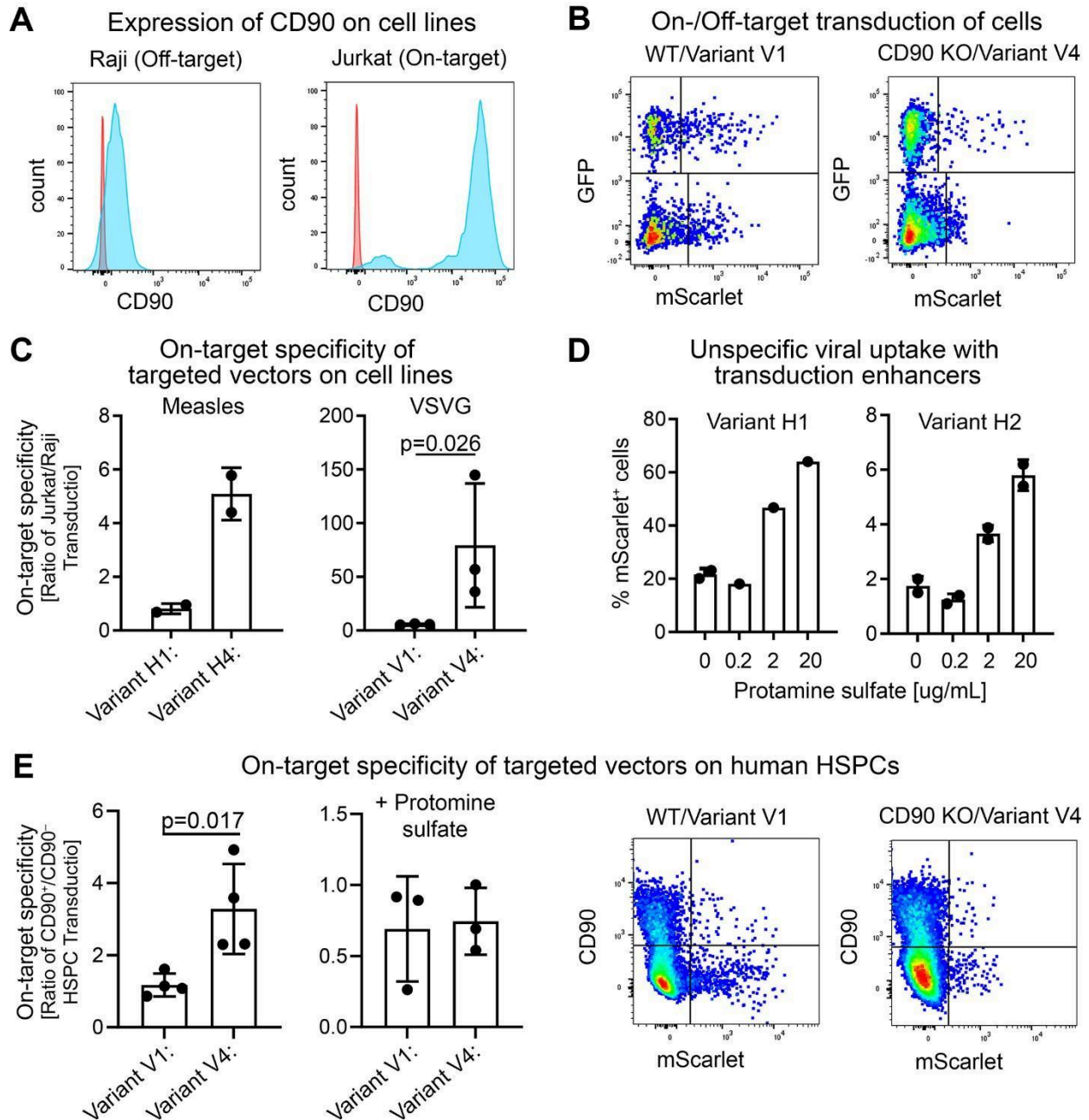


Figure 2: On-target specificity of CD90-targeted vectors.

(A) Flow-cytometric assessment of CD90 expression on Raji and Jurkat cells. (B) Transduction efficiency of mixed cultures containing GFP⁻ Raji and GFP⁺ Jurkat cells with either Variant V1 or V4. (C) On target specificity of Measles- and VSV-G-based vector variants 1 and 4 determined by the ratio of mScarlet⁺GFP⁻ cells within total GFP⁻ Raji to mScarlet⁺GFP⁺ cells within total GFP⁺ Jurkat cells. (D) Dose-dependent impact of the transduction enhancer protamine sulfate

on the transduction of Jurkat cells with engineered measles-based vector variants 1 and 2. (E) On target specificity of VSV-G-based vector variants 1 and 4 determined by the ratio of mScarlet⁺CD90⁻ cells within total CD34⁺ cells to mScarlet⁺CD90⁺ cells within total CD34⁺ cells in the absence and presence of protamine sulfate. (Statistics: Mean±SD, T-test, significance as indicated)

2.5 Tables

Table 1

Antigen	Vendor	Catalog Number	Clone Name	Fluorochrome	Application
CD45	BD Biosciences	560367	HI30	V450	Human HSCs
CD34	BD Biosciences	561209	563	APC	Human HSCs
CD90	BD biosciences	328124	5E10	PE-Cy7	Human HSCs, Jurkat cells Raji Cells

2.6 Materials and Methods

Cloning of virus plasmids

VSV-G Knockout (KO) and targeted binder plasmids were provided by the Baker Laboratory (Institute of Protein Design, University of Washington, Seattle, WA). Cloning of CD90 scFv into the targeted binder plasmid was performed by Eco721-FastDigest (Thermo Fisher Scientific, cat # FD0364) and Eco1471-FastDigest (Thermo Fisher Scientific, cat # FD0424) restriction enzyme digest of targeted binder plasmid followed by Gibson Assembly (NEB, cat # E2611S) with synthesized Gibson block sequence (Integrated Data Technologies, Coralville, IA) containing the CD90 scfv sequence. Successful cloning was confirmed by EcoRI (NEB, cat # R3101S) restriction enzyme digest, followed by sequencing on Applied Biosystems 373xl DNA Analyzers (Thermo Fisher Scientific).

Virus production

HEK293T cells were expanded on gelatin coated plates and transfected with plasmids mixed with PEI (Polysciences, USA, PA) at a $\frac{1}{4}$ μg plasmid to μg PEI ratio. Measles virus plasmid ratios were 27 μg pLenti-EF1a-mScarlet-I-WPRE transfer plasmid, 17 μg 2nd generation Gag/Pol Helper plasmid pCMVdelta8.74, 10 μg measles hemagglutinin Delta 24H, and 10 μg measles fusion Delta F per 15 cm cell plate. VSV-G virus plasmid ratios were 27 μg pLenti-EF1a-mScarlet-I-WPRE transfer, 17 μg 2nd generation Gag/Pol Helper plasmid pCMVdelta8.74, 10 μg VSV-G fusion, and 10 μg of relevant targeting ligand plasmid per 15 cm cell plate. Twenty-four (24) hours after transfection, the media was changed to LV-MAX (Thermo Fisher Scientific) media with 20 mM HEPES (Thermo Fisher Scientific) and 1X L-glutamine (Thermo Fisher Scientific). Virus production media was allowed to sit for a further 24 hours, then the supernatant was collected, spun down at 800 g to remove cellular debris, filtered with an 8 μm filter, and spun down for 24 hours at 4800 g. The concentrated supernatant was resuspended at a 1/100 volume.

Hydrodynamic titration

Hydrodynamic titration of viral particles was performed on a Zetaviewer machine (Particle Metrix, GmbH, Inning am Ammersee, Germany). The machine was calibrated with a 100 nm bead test sample prior to all measurements. Viruses were diluted 1 to 1000 in molecular grade water and analyzed for their size characteristics. Readouts from the machine were back calculated to account for dilution. Diluted virus samples were next incubated with 1x SYBR II Green RNA stain (Thermo Fisher Scientific) for 5 minutes before running on the Zetaviewer with a 488 nm filter in place to only identify virus particles loaded with RNA and stained with SYBR II. Additionally, virus's targeted to CD90 were incubated with SYBR II and CD90ex protein for 30 minutes. Virus-protein complexes were analyzed on the Zetaviewer with a 488 or 520 nm filter in place to capture particles loaded with RNA carrying CD90ex.

Cell lines

Jurkat (ATCC CLR-2899) cells, Raji (ATCC CCL-86), and K-562 (ATCC CCL-243) cells were all cultured in RPMI with Penicillin Streptomycin at a final concentration of 50 to 100 I.U./mL (Thermo Fisher Scientific) and supplemented with 10% cosmic calf serum (Thermo Fisher Scientific).

Primary human HSCs

Primary human CD34⁺ cells were purchased from the Co-operative Center for Excellence in Hematology (CCEH) at the Fred Hutchinson Cancer Center (Fred Hutch). Collections were performed according to the Declaration of Helsinki and were approved by a local ethics committee/institutional review board of Fred Hutch. All healthy adult donors were mobilized with GCSF. Human CD34⁺ cells were enriched as previously described on a CliniMACS Prodigy according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

Human CD34⁺ cells were cultured in StemSpan (STEMCELL Technologies, Vancouver, BC, Canada) medium supplemented with penicillin-streptomycin (PS) (100 U/mL) (Gibco by Life Technologies, Waltham, MA, USA) and 100 ng/mL of each stem cell factor (PeproTech, Cranbury, NJ, USA), thrombopoietin (TPO; PeproTech), and Fms-related tyrosine kinase 3 ligand (FLT3-L; Miltenyi Biotec). Cells were cultured at 37°C, 85% relative humidity, and 5% CO₂.

Virus transduction

Cells (Jurkat, Raji, human HSCs) were resuspended in serum-free media at a density of 150,000 cells per 150 µL per well of a 96-well plate (Corning, Corning, NY, USA). Concentrated viral supernatant was added to the cells and incubated for 8-12 hours. After transduction cells were washed and media replaced with relevant growth media specific to cell type (see above). Transgene expression was determined flow-cytometrically 72-120 hours post-transduction.

Flow cytometry analysis and cell sorting

Flow cytometric analysis and sorting of cell lines and human CD34⁺ cells were performed using the fluorochrome-conjugated antibodies listed in **Table 1**. Dead cells and debris were excluded via forward light scatter (FSC)/side light scatter (SSC) gating and DAPI staining. Flow cytometric analysis and cell sorting were performed on a FACSymphony A5, FACSCelesta, FACS Aria IIu, and Symphony S6 (BD Biosciences, San Jose, CA). Data were acquired using FACSDiva version 6.1.3 and newer (BD Biosciences). Data analysis was performed using FlowJo version 8 and higher (BD Biosciences).

Chapter 2: In Vivo Targeting of CD90⁺ HSCs Enables Multiplexed Gene Modification

2.1 Introduction

Defining robustly expressed and HSC-enriched antigen targets is crucial for precisely targeting HSCs for gene and cellular therapies in vivo. In previous studies, we and others have defined key surface antigens that can enrich an HSC-containing subset responsible for long-term hematopoietic reconstitution [10]. In humans and the nonhuman primate (NHP) model, this subset can be defined by the surface antigen phenotype of CD34⁺CD45⁺CD90⁺CD45RA[−] (CD34⁺CD90⁺ HSCs) [11, 44, 45]. Alternatively, CD133, CD164, CD202 (EPCR), CD33, and CD117 have been used to phenotypically identify human HSC markers [46-52]. Most important for HSC gene therapy, direct lentiviral transduction of FACS-purified CD34⁺CD90⁺ cells ex vivo resulted in improved gene-modification efficiency of human HSCs, with long-term multilineage engraftment potential in NSG mouse xenograft transplants [11]. To overcome the logistical challenges associated with the FACS-purification of CD34⁺CD90⁺ HSCs and enable direct targeting of cells ex vivo, we recently developed novel lentiviral gene therapy vectors with increased specificity for HSCs in vitro by studding the vesicular stomatitis G (VSV-G) and measles virus envelopes with an anti-CD90 single-chain variable fragment (scFv) [53]. Based on these works, we report a novel viral particle platform for in vivo HSC gene therapy. CD90-targeted viral particles injected via intraosseous or intravenous injection into humanized NBSGW mice enabled transduction of a bona fide HSC population as validated by gene marking in secondarily transplanted immunodeficient mice. Furthermore, utilizing the CD90-targeting strategy, we achieved in vivo editing of the HBG1/2 promoters, leading to the reactivation of fetal hemoglobin in CD34-derived erythrocytes.

2.2 RESULTS

2.2.1 Highly variable and collection dependent abundance of potential antigen targets for HSC-directed gene therapies

We compared the expression and surface abundance of common HSC antigens utilizing a combination of bulk/single cell RNA-sequencing (scRNAseq) and quantitative flow cytometry in human and nonhuman primate (NHP) steady-state BM (ssBM) CD34+ cells (ssBM). Analysis of surface antigen transcripts showed high CD34, CD45, and CD164, low/inconsistent expression for CD133, and very low to undetectable expression for CD33, CD90, CD117, and EPCR in bulk as well as single cell datasets across species (Fig. 1a-b, Fig. S1). As seen by RNAseq, quantitative flow-cytometry showed high abundance of CD34, CD45, and CD164 (>10K antigens/cell) and low abundant of CD133, CD117, and CD33 (2,586/1,085/905 antigens/cell) (Fig. 1c). Despite the lack of detection by RNA-seq, surface protein expression was found for CD90 and EPCR (3,110/263 antigens/cell). Abundance of the cross-reactive antigens CD90, CD117 and CD164 in the NHP mirrored the human data (Fig. S2a-b).

Mobilization of HSCs into circulation followed by modification via intravenous (IV) administration of gene therapy agents is currently considered the most accessible and least invasive treatment route¹⁸⁻²⁰. Here, we compared the HSC antigen mRNA expression (bulk and scRNAseq) as well as surface presentation on human and NHP CD34+CD90+ HSCs collected from mobilized apheresis (APH) products and GCSF-primed BM (pBM)-HSCs (only available in the isogenic NHP setup) to HSCs from ssBM (Fig. 1c, Fig. S2a-d). CD33, CD34, CD45, CD90 and EPCR cell surface expression levels were independent of collection methodology, whereas a reduction in CD164 as well as CD117 and an increase in CD133 antigens was seen in APH compared to ssBM (Fig. 1c, Fig. S2b). ssBM-HSCs showed a homogenously high expression of CD164 in

contrast to APH- and pBM-HSCs, which displayed three distinct populations of CD164^{hi}, CD164^{med}, and CD164^{lo} HSCs, indicating a G-CSF-induced loss of CD164 on G-CSF-treated and mobilized HSCs (Fig. S3). We similarly observed a change in the CD117 distribution on HSCs from predominantly CD117^{hi} HSCs (87.6%) in the ssBM to a heterogeneous mix of CD117^{hi} (52.9%) and CD117^{lo} (47.1%) populations in APH and a 10-fold higher proportion of CD117^{hi} HSCs in pBM products, suggesting a more preferential mobilization of CD117^{lo} cells into the periphery after G-CSF administration (Fig. S3). Thus, mobilized HSCs and non-mobilized HSCs do not homogeneously share antigen abundance. Of the antigens tested, however, CD33, CD34, CD45, CD90, and EPCR were unchanged after G-CSF treatment in human samples.

Finally, we determined the absolute and relative antigen abundance of target antigens on HSCs in order to calculate the competition and number of non-HSC antigen sinks in vivo gene therapy agents will experience. CD34 had the highest antigen abundance on HSCs, with 16.5% of all CD34 antigens measured found in the HSCs cluster, followed by CD90 (10.2%), CD117 (7.37%), CD164 (4.43%), CD133 (0.39%), CD33 (0.98%), EPCR (0.59%) and CD45 (0.39%) (Fig. 1d, Fig. S4a). CD117 staining was found on CD34⁺ hematopoietic progenitor cells (HPCs), CD4⁺ CD8⁺ T cells as well as on non-lymphocyte populations (CD45^{neg}) (Fig. S4b). CD90 was predominantly found on the HSPC population and a subset of non-lymphocytic cells. In summary, CD90 expression is not only more restricted to HSCs in the bone marrow but also 2- to 3-fold higher expressed on our target cells than CD117.

2.2.2 Targeted coccal-pseudotyped lentivirus improves HSC transduction in vitro and in vivo

Based on the results above reaffirming the utilization of CD90 as a specific target for HSCs, we aimed to build on our previous work designing novel targeted lentiviral particles (LV) for in vivo

applications¹⁷. The coccal virus envelope shares 75% of the amino acid sequence with the VSV-G envelope while maintaining its broad tropism. Additionally, coccal-pseudotyped lentiviral particles have increased serum resistance, improved HSC transduction capacity, and increased viral titers in manufacturing^{21, 22}. We hypothesized that a CD90-targeted coccal-pseudotyped LV would be more suitable for in vivo HSC gene therapy applications. We performed a similar amino acid substitution as in the previously reported LDL-R tropic ablation VSV-G mutants to produce a coccal envelope variant unable to bind to LDL-R^{17, 23}. To evaluate the HSC targeting specificity of the novel CD90-targeted coccal-pseudotyped lentivirus (Cocal-CD90), we treated G-CSF mobilized CD34⁺ HSPCs with an equal number of WT-Cocal and Cocal-CD90 LVs. Cocal-CD90 transduced CD34⁺CD90⁺ HSCs with significantly greater specificity than WT-Cocal ($p=0.0008$) but with similar transduction efficiency (Fig. 2a). Next, we compared Cocal-CD90 with our VSV-G-CD90 for transduction efficiency and specificity on G-CSF mobilized CD34⁺ HSPCs. Cocal-CD90 transduced target HSCs at 2.12-fold increased efficiency in comparison to VSV-G-CD90 ($p=0.017$) (Fig. 2b), though they maintained comparable on-target HSC specificity ($p=0.118$) (Fig. 2a).

The NBSGW xenograft murine model can be utilized to evaluate targeting efficiency, specificity, and route of administration of novel HSC gene therapy platforms in vivo^{24, 25} (Fig. 2c). We tested whether Cocal-CD90 LVs could deliver the mScarlet fluorescent transgene to long-term human HSCs in vivo while maintaining hematopoietic differentiation capacity and low-off target transduction. Viral particles were delivered via intraosseous (IO) injection to three independent cohorts of 10- to 12-week-old mice engrafted with CD34⁺ cord blood from three separate donor pools. After 6 weeks post-LV injection, mice were necropsied, and bone marrow from the injected bone was analyzed for mScarlet⁺ signaling by flow cytometry. We observed positive mScarlet⁺ signaling in human-specific CD45⁺ cells in all mice injected with Cocal-CD90 LV.

Moreover, 88% of all mScarlet+ cells were positive for CD90 expression confirming selective transduction of on- and off-target cells expressing the targeted antigen (Fig. 2d).

To test the ability of Cocal-CD90 to transduce human HSCs in vivo, we compared the mScarlet signal in phenotypic HSPC subsets in humanized mice receiving either WT- or CD90-LVs. As seen ex vivo, Cocal-CD90 transduced CD34+CD45+CD38–CD90+ cells (HSCs), CD34+CD45+CD38– (MPP), and more general CD34+ HSPCs, while barely any transduction was seen in phenotypic HSPC subsets using the Cocal-WT LV (Fig. 2e). Furthermore, transduced human HSPCs showed contribution to myeloid and lymphoid lineages in the bone marrow (Fig. 2f).

In summary, Cocal-pseudotyped viral vectors can be similarly mutated to lose their naïve LDL-R binding capability and be decorated with our CD90 targeting molecules to specifically transduce human HSCs ex vivo as well as in vivo in humanized NBSGW mice.

2.2.3 Derivation of a novel CD90-binder for improved in vivo transduction

To further improve our CD90-VP platform, we employed crystallography of the anti-CD90 antibody (Fig. 3a, Supplemental Table 1), identified an alternate variable light chain (VL) sequence (Fig. 3b) and engineered an alternate second-generation (2nd-gen) anti-CD90 scFv for incorporation into the Cocal-CD90 LV. We further implemented the canonical G4S linker in our 2nd-gen scFv26, 27. Finally, we compared 1st- and 2nd-gen CD90 scFv-decorated Cocal LVs in humanized NBSGW mice to assess transduction efficiency and on-target specificity (Fig. 3c, Fig. S5).

A 2-fold greater transduction efficiency of human CD34+CD90+ HSCs was seen when mice were injected IO with our new 2nd-gen Cocal LVs compared to the 1st-gen counterpart (Fig. 3d).

Similarly, IV administration of Cocal LVs without prior mobilization led to a 3-fold greater transduction efficiency of human CD34+CD90+ HSCs with 2nd-gen Cocal LVs in comparison to 1st-gen LVs (Fig. 3e). Equivalent on-target specificity was seen after IV and IO administration with both 1st- and 2nd-gen LVs (Fig. 3f).

To confirm successful gene-modification of long-term engrafting HSPCs with CD90-targeted LVs, CD34+ HSPCs from the BM of in vivo transduced mice were FACS-purified and transplanted into the femur of low-dose busulfan-conditioned adult NBSGW mice to assess secondary engraftment potential (Fig. 3c). Twelve to fourteen weeks after transplantation, mScarlet expression was seen within engrafted human CD45+ cells (Fig. 3f, Fig. S5c). Greater mScarlet frequencies were observed for mice receiving 2nd-gen LVs, mirroring trends observed in primary transplants. Of note, transduction rates were 10- to 20-fold higher than initially observed in primary transplants, suggesting inefficient transgene expression in successfully transduced, quiescent human CD34+CD90+ HSCs in primary mice.

2.2.4 In vivo gene-editing of HSPCs with CD90-VLPs reactivates fetal hemoglobin

The inhibition of BCL11A expression or its binding to the HBG1 and HBG2 (HBG1/2) promoters reactivates fetal hemoglobin expression in erythrocytes and ameliorates the pathophysiology of sickle cell disease (SCD) [54]. This biological basis is the strategy of multiple Cas9-mediated gene-modification therapeutics in recently approved ex vivo HSC gene therapy products [55]. However, ex vivo HSC gene therapy requires sophisticated infrastructure inaccessible to many patients in low- and middle-income countries. Recent efforts in the field focus on loading lentiviral particles with Cas9 protein to enable concomitant gene editing and transduction of cells in vivo directly in the patient [56, 57].

We thus sought to explore whether our CD90-Targeted Cocal LVs can deliver Cas9 to HSCs in vitro and in vivo. 2nd-gen Cocal-CD90 LVs were loaded with Cas9 protein (Cocal-CD90-Cas9) together with the previously reported HBG-113 gRNA29, 32 under U6 promoter regulation. Freshly thawed G-CSF mobilized human CD34⁺ HSPCs were treated ex vivo with Cocal-CD90-Cas9 LVs, cultured for three days; next, CD90⁺ and CD90[−] HSPCs were FACS-purified and then analyzed for vector copy number (VCN) as well as HBG promoter editing (Fig. 6a). The VCN within CD34⁺CD90⁺ HSCs was significantly higher than in CD34⁺CD90[−] progenitors, with an average of 0.96 copies per cell in CD34⁺CD90⁺ cells compared to 0.12 copies per cell in CD34⁺CD90[−] cells ($p=.0159$) (Fig. 6b). To analyze HBG editing, Sanger sequencing of the HBG1/2 promoter locus was performed and data analyzed with TIDE33. 66.33% of HBG 1/2 promoter alleles in CD34⁺CD90⁺ HSCs contained indels, while only 12.27% of HBG 1/2 promoter alleles in CD34⁺CD90[−] progenitors showed indels (Fig. 6c). Of note, transduction as well as editing in CD34⁺CD90[−] cells is presumed to be a result of successfully transduced and differentiating CD34⁺CD90⁺ HSCs during the 3-day culture rather than off-target transduction. To validate the functionality of HBG promoter engineering, we differentiated FACS-purified CD34⁺CD90⁺ HSCs and CD34⁺CD90[−] progenitors into erythroid cells. Assessment of fetal hemoglobin reactivation by flow cytometry confirmed that engineered CD34⁺CD90⁺ HSCs displayed, on average, a 30% increase in fetal hemoglobin compared to CD34⁺CD90[−] progenitors (Fig. 6d).

We next explored whether IO administration of Cocal-CD90-Cas9 particles into humanized NBSGW allows HSC engineering of the HBG promoters directly in vivo (Fig. 6e). NBSGW mice were injected with Cocal-CD90-Cas9 into one femur, whereas the other limb served as an internal control. Seven days after injection, BM from both limbs was collected, and human CD34⁺ HSPCs were FACS-purified and expanded in vitro for an additional seven days before analysis for VNC and HBG editing by Sanger sequencing. A VCN of 0.18 (Fig. 6f) and 30.3 %

HBG editing (Fig. 6g) were seen in the injected bone of mice receiving Cocal-CD90-Cas9 LVs, closely matching the previously observed transduction efficiency of our CD90-targeted LVs in secondary mouse transplants. To confirm the maintenance of erythro-myeloid differentiation potential, CD34+ HSPCs from control (non-injected) as well as IO-injected bone marrow were introduced into CFC assays (Fig. 6h), showing no significant differences in the total number and type of colonies.

2.3 Discussion

We report the development of a CD90-targeted genetic engineering platform that allows for the specific gene modification of CD34+CD90+ HSCs both in vitro and in vivo. Analysis of potential HSC-specific antigen markers via both RNA transcriptomic and quantitative flow analysis confirmed CD90 protein as a highly abundant antigen on HSCs. As such, we further pursued the development of adapting a CD90-targeting binder on the cocal viral envelope. These improved Cocal-CD90 envelopes showed superior viral transduction efficiency in vitro and in vivo while maintaining specificity towards CD90+ cells. Re-engineering of the CD90 binding motif further increased the in vivo transduction efficacy of HSCs independent of the VP administration route. As a pre-clinical model of the utility of this CD90-targeted genetic engineering platform, we loaded CD90-VPs with Cas9-gRNA ribonucleotide proteins targeted against the BCL11A binding site on the HBG 1 and HBG 2 promoter. Cocal-CD90^{2nd}-Cas9 concomitantly transduced and edited CD90+ cells in vitro, leading to therapeutic levels of fetal hemoglobin within edited erythroid cells.

The in vivo administration of gene engineering modalities is needed to improve the accessibility of gene and cellular therapies worldwide [58]. Numerous platforms are in development that can deliver gene-editing nucleases or therapeutic transgenes to cells of interest [19, 59-63]. However, significant benchmarks must be met to provide such strategies in the clinic. These include high tissue specificity, low immunogenicity, and iterative capacity [58].

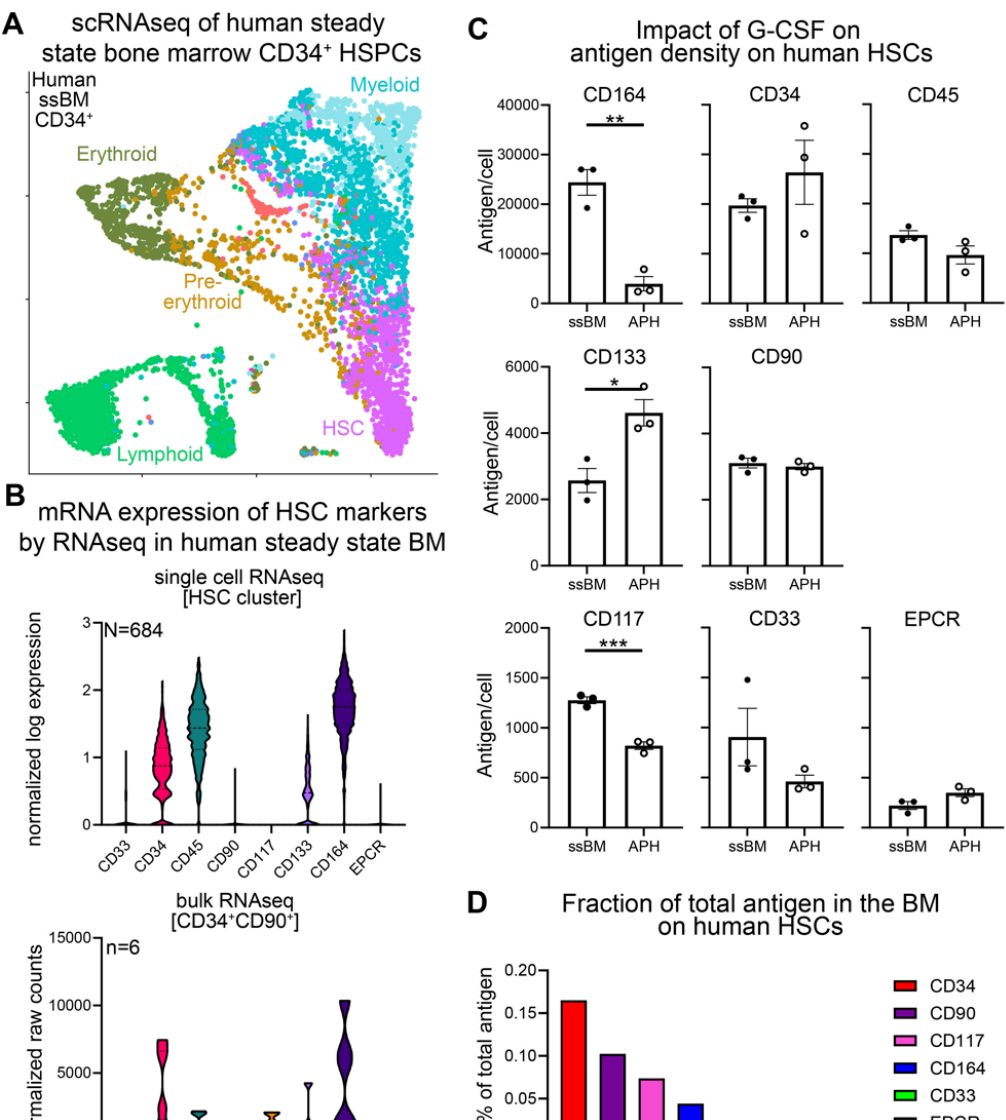
While lipid nanoparticles (LNPs) are the most advanced in displaying robust gene modification, they have lacked tissue specificity [64]. This deficit can be overcome through local injection or the addition of targeting moieties. However, such modifications have only minimally decreased the natural hepatic tropism of LNPs [60, 61]. In contrast, utilizing the Cocal-CD90 platform, we observed over 70% of transgene-positive cells in vivo to additionally express our target antigen. Furthermore, contemporary studies utilizing the cocal envelope in the NHP model find no evidence of significant off-target organ transduction with engineered cocal envelopes [62]. Adeno-associated virus (AAV) and helper-dependent adenoviruses (HDA_d) in vivo platforms improve upon LNPs in their tissue specificity but remain immunogenic, requiring immunosuppressive prophylaxis. Furthermore, AAVs lack iterative capacity as cell-specific serotype tropism requires new library screens for novel antigen targets [65]. Our platform is highly iterative, as tropism is determined by scFv binding. Moreover, the Cocal-CD90 platform enables multiplexing gene modification strategies with the potential to insert novel transgenes and gene-edit pathological alleles. Unlike other platforms, this allows for robust parallel administration of multiple genetic modifications that have the potential for synergistic physiology. For instance, combinatorial strategies of delivering anti-sickling hemoglobin while reactivating fetal hemoglobin through Cas9 editing modalities may be an innovative treatment strategy for SCD utilizing Cocal-CD90-2nd-Cas9 [66-68]{Samuelson, 2021 #182}.

Even with these substantial advancements, the Cocal-CD90 platform could be improved through further innovations. We observed a broad distribution of in vivo editing efficiencies, though the same number of particles were administered. Our editing efficiencies ranged from 4.6% to 46% in mice, suggesting that the recipient's physiology may innately determine editing. As such, post-gene therapy enrichment strategies to increase gene modification above therapeutic thresholds may be necessary in scenarios of inefficient gene editing. Such methods are currently employed in preclinical models to provide a proliferative advantage of

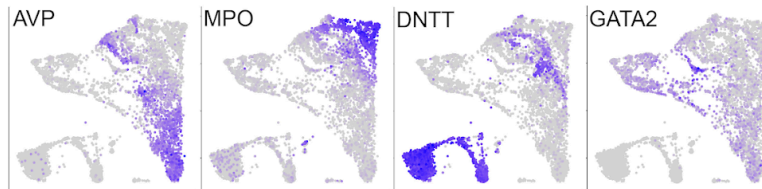
gene-modified HSCs or remove unedited HSCs via chemoselection, CAR-T cells, or antibody-drug conjugates (ADCs) [69-73]. These platforms could significantly improve the feasibility of in vivo gene therapy and mark a significant future direction for the field.

In conclusion, the Cocal-CD90 platform allows for the co-delivery of transgenes and gene-editing nucleases within HSCs. The delivery strategy had no measurable effect on hematopoietic differentiation capacity or self-renewal. Thus, Cocal-CD90 has broad future implications in providing a more accessible therapeutic delivery platform for treating not only hemoglobinopathies but also many other genetic diseases that are currently being treated or considered for HSC gene therapy or allogeneic transplantation.

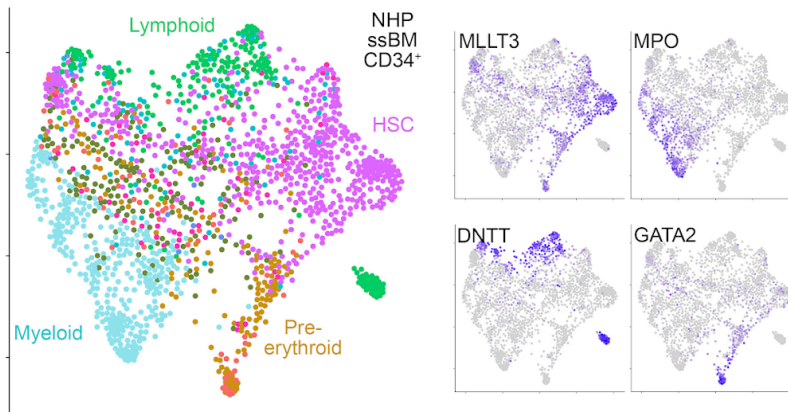
2.4 Figures and Legends



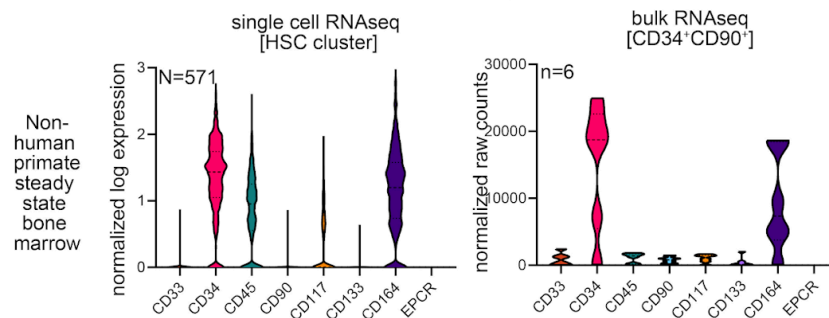
A scRNAseq of human steady state bone marrow CD34⁺ HSPCs



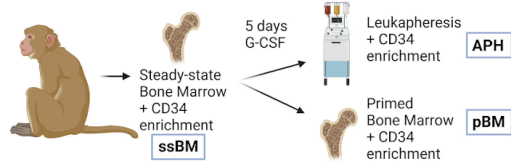
B scRNAseq of NHP steady state bone marrow CD34⁺ HSPCs



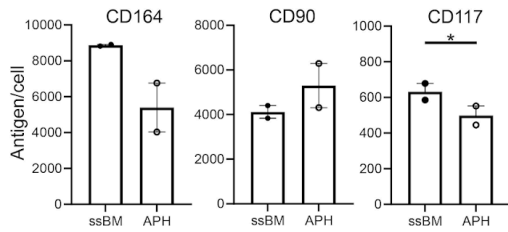
C mRNA expression of NHP HSC markers by RNAseq in steady state



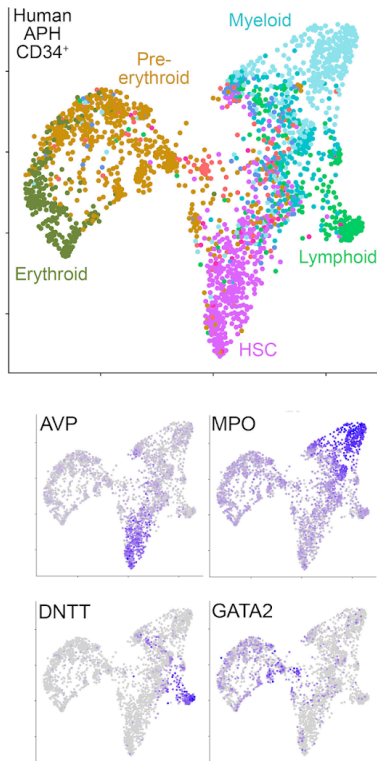
A Isogenic assement of antigens in the NHP



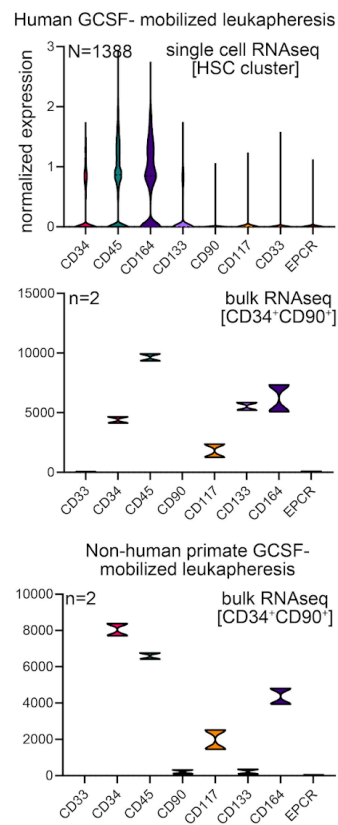
B Impact of G-CSF on antigen density on NHP HSCs

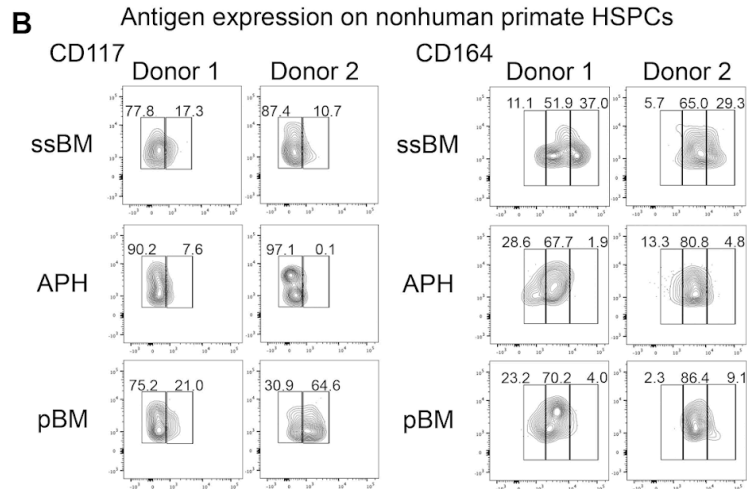
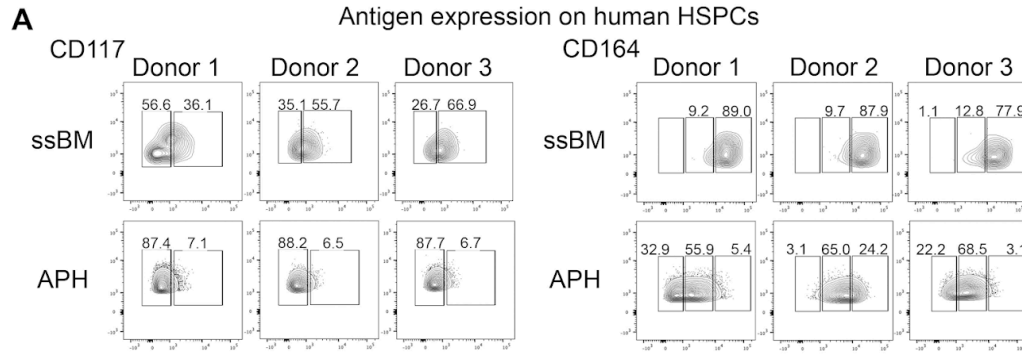


C scRNAseq of human GCSF-mobilized human CD34⁺ HSPCs

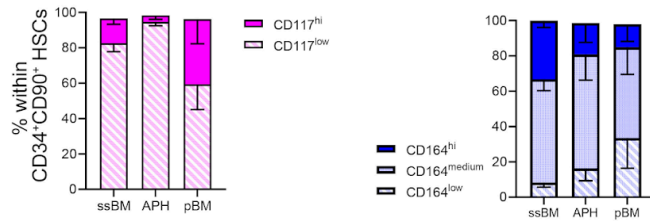


D mRNA expression of HSC markers by RNAseq after mobilization

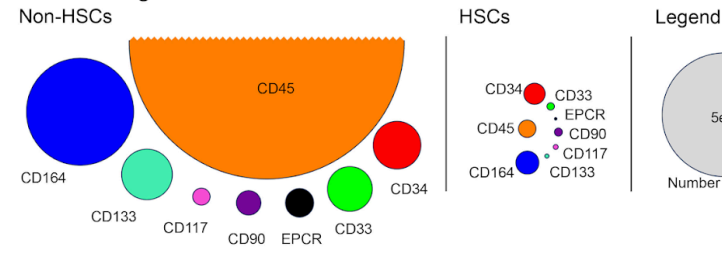




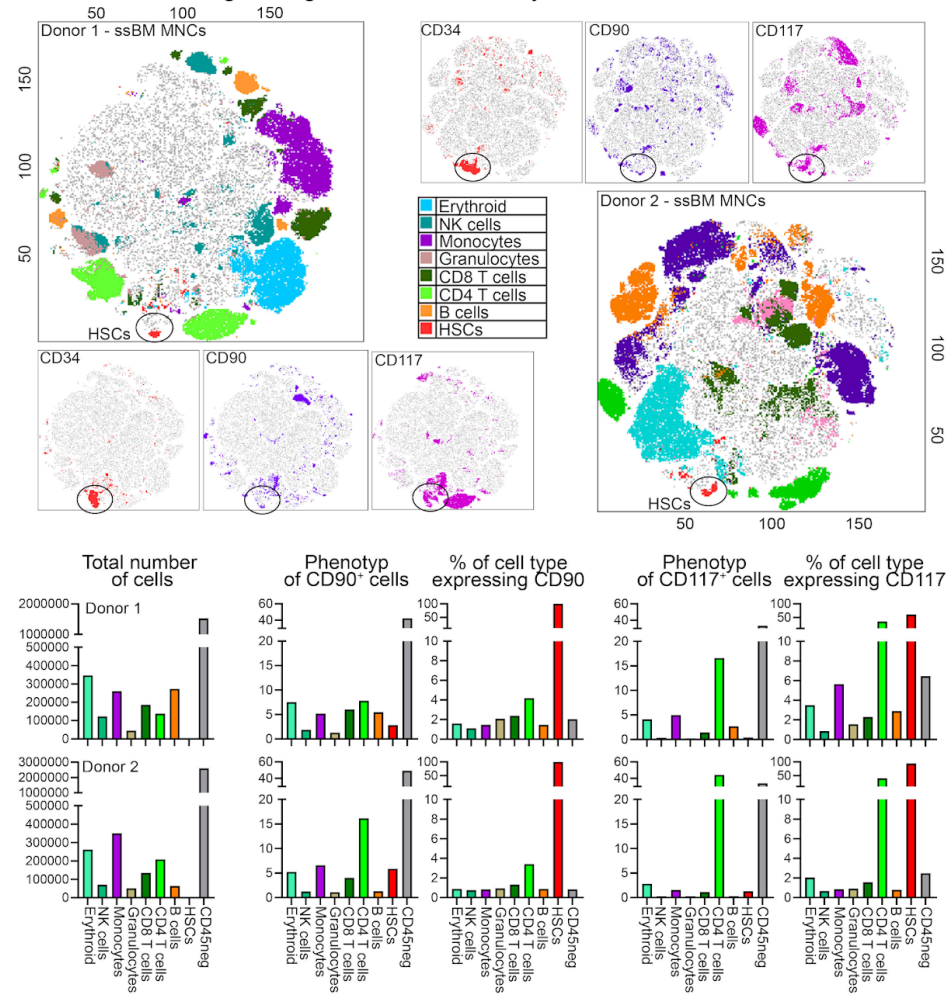
C Phenotypic composition in ssBM, pBM, and APH HSPCs in NHPs

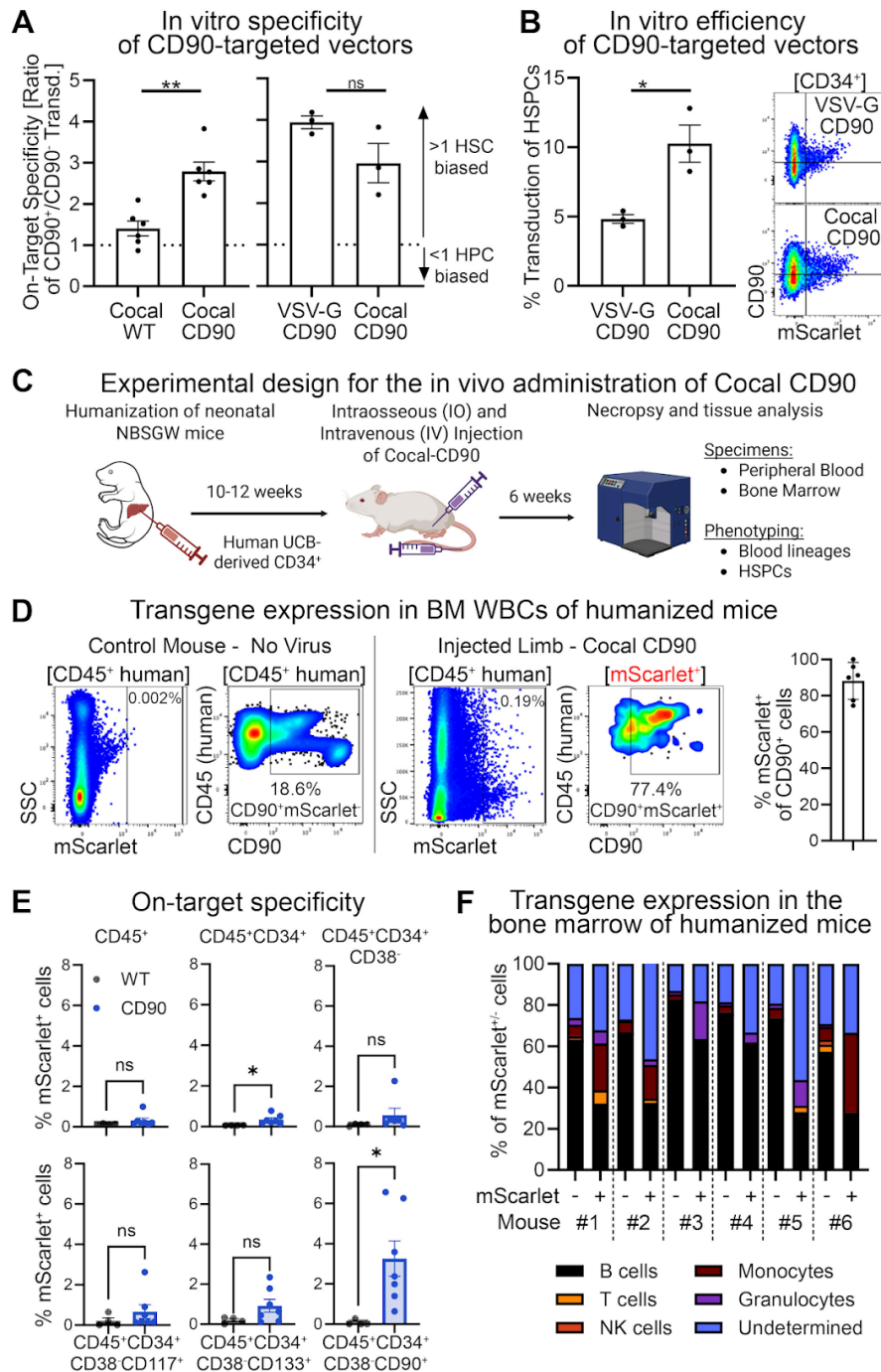


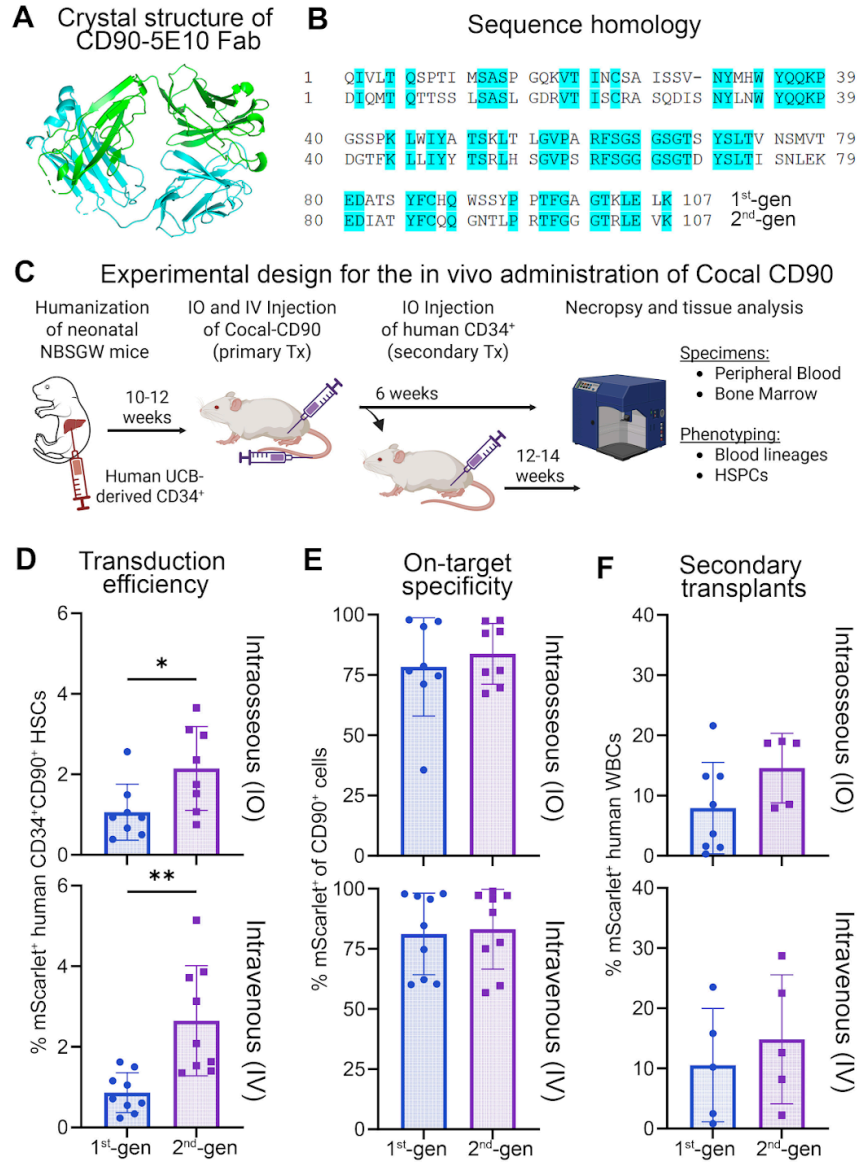
A Antigen distribution in ssBM

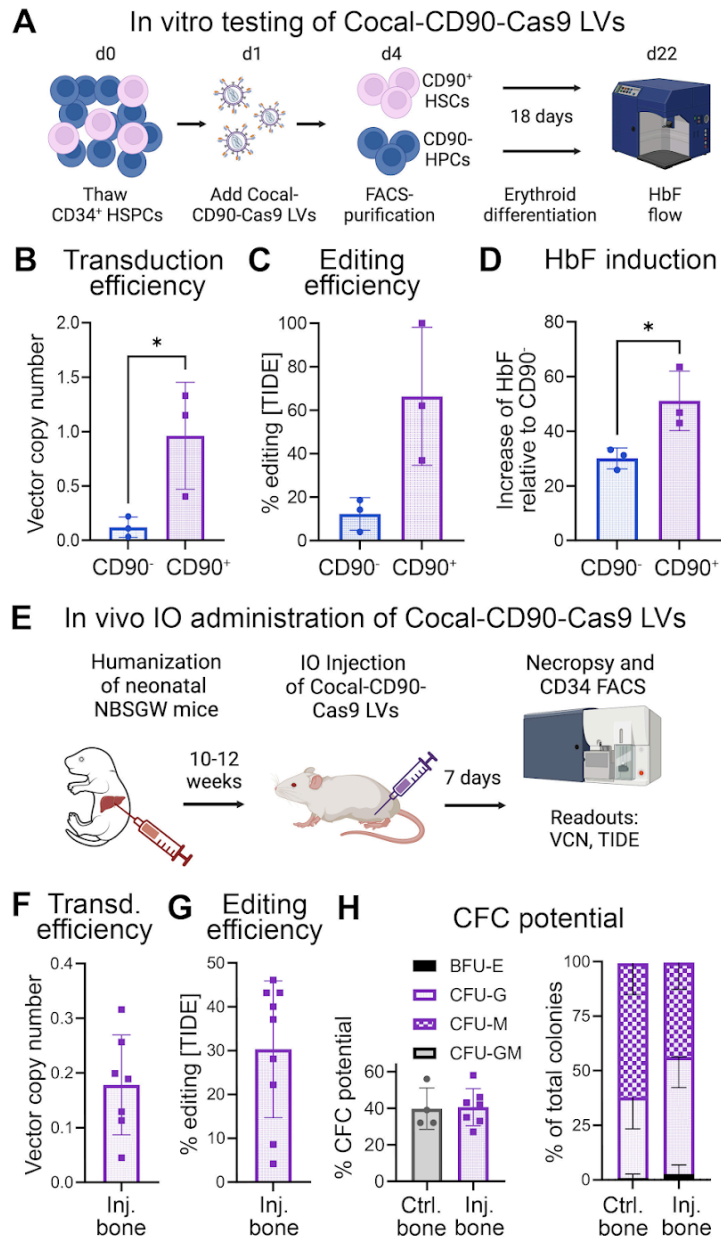


B Off-target antigens in human steady state bone marrow WBCs









2.4 Tables

2.5 MATERIALS AND METHODS

Primary human and nonhuman primate HSPCs

Primary human CD34⁺ cells and BM aspirates were purchased from the Co-operative Center for Excellence in Hematology (CCEH) at Fred Hutchinson Cancer Center (Fred Hutch). Collections were performed according to the Declaration of Helsinki and were approved by a local ethics committee/institutional review board of Fred Hutch. All healthy adult donors were mobilized with G-CSF. Human CD34⁺ cells were enriched on a CliniMACS Prodigy according to manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany).

For NHP experiments utilizing mobilized peripheral blood, G-CSF was administered once daily at 50 µg/kg/day for 5 days to pigtail macaques. Before enrichment for NHP CD34⁺ cells, red cells were lysed twice in ammonium chloride lysis buffer, and the remaining white blood cells were incubated for 25 min with 40 µg per 1×10⁸ cells of custom-produced 12.8 IgM anti-CD34 antibody (Fred Hutch, Seattle, WA). The cells were then washed and incubated for a further 25 min with 1 mL per 1×10⁸ cells of magnetic-activated cell-sorting anti-IgM microbeads (Miltenyi 130-047-301). After a second wash, the cell suspension was run through magnetic LS Columns (Miltenyi 130-042-401) enriching for CD34⁺ cell fractions with a maximum of 2×10⁹ cells per column and an initial purity of 14%–52% confirmed by flow cytometry.

Human and NHP CD34⁺ cells were cultured in StemSpan (STEMCELL Technologies, Vancouver, BC, Canada) medium supplemented with penicillin-streptomycin (PS) (100 U/mL) (Gibco by Life Technologies, Waltham, MA, USA) and 100 ng/mL of each stem cell factor (PeproTech, Cranbury, NJ, USA), thrombopoietin (TPO; PeproTech), and Fms-related tyrosine kinase 3 ligand (FLT3-L; Miltenyi Biotec). Cells were cultured at 37°C, 85% relative humidity, and 5% CO₂.

Viral Particle production

Targeted lentiviral production was performed as described¹⁷. HEK293T cells were plated on gelatin-coated plates and transfected with plasmids mixed with PEI (Polysciences, USA, PA) at a 1/4 µg plasmid to µg PEI ratio. VSV-G or Cocal virus plasmid ratios were 22 µg gene transfer payload (pLenti-EF1a-mScarlet-I-WPRE or U6_HBGgRNA), 22 µg 2nd generation Gag/Pol Helper plasmid pCMVdelta8.74, 11 µg VSV-G or Cocal KO envelop plasmid, and 22 µg of relevant targeting ligand plasmid (V2 or V4) per 15 cm cell plate. For the production of VLPs, an additional 44 µg of Gag-Cas9 (pJRH029) plasmid was utilized. pJRH029 was a gift from Jennifer Doudna (Addgene plasmid # 171060 ; <http://n2t.net/addgene:171060>; RRID:Addgene_171060). Twenty-four (24) hours after transfection, the media was changed to DMEM media with 10% Cosmic Calf Serum, 1mM Penn/Strep, and 1X L-glutamine (Thermo Fisher Scientific). Virus production media was allowed to sit for a further 24 hours, and then the supernatant was collected, spun down at 800 g to remove cellular debris, filtered with a 0.45 µm filter, and spun down for 24 hours at 5000 g. The concentrated supernatant was resuspended at 1/100th of the original volume. As previously described, hydrodynamic titration of viral particles was performed on a Zetaviewer machine (Particle Metrix, GmbH, Inning am Ammersee, Germany)¹⁷.

In Vitro Viral Particle Delivery

CD34+ HSPCs were resuspended in serum-free media at a density of 150,000 cells per 150 µL per well of a 96-well plate (Corning, Corning, NY, USA). Concentrated viral supernatant was added to the cells and incubated for 8-12 hours. After transduction, cells were washed, and the media was replaced with SFEMII media. Transgene expression was determined flow-cytometrically 72 hours after transduction.

Xenotransplantation of NBSGW mice and In Vivo Delivery of Viral Particle

NBSGW neonates were humanized, as previously discussed by Choo et al. (2024)⁵¹. Briefly, NBSGW neonates aged 1 to 3 days were intrahepatically injected with 30 000 to 50 000 human umbilical cord blood (UCB)-derived CD34⁺ HSPCs per animal in 30 μ L of injection media (filtered RPMI 1640, 1 μ M EDTA). Starting at eight weeks after injection, human chimerism was measured as the percentage of human CD45⁺ cells among the mouse and human CD45⁺ in the PB. Blood analysis was performed every other week until necropsy, at which BM, PB, and spleen were collected.

Ex Vivo Erythroid Differentiation

Differentiation of HSPCs was performed utilizing a previously published protocol described⁵². Differentiation was performed by timed addition of hematopoietic growth factors and cytokines in basal erythroid differentiation media (EDM). Stage I media was composed of EDM + IL-3 (5 ng/mL) + SCF (100 ng/mL) + Erythropoietin (EPO) (6 U/mL). Stage II of differentiation was initiated by seeding 200,000 cells per well of a 24-well plate in Stage II media for 4 days. Stage II media was composed of EDM + SCF (50 ng/mL) + EPO (6 U/mL). For Stage III, 500,000 cells were seeded into EDM + EPO (2 U/mL) for 8 days. Erythroid differentiation was confirmed by flow cytometry analysis of positive staining with CD71 and CD235a.

Editing Efficiency Determination

Allele editing frequency was determined as described by Humbert et al. (2021)³². Extraction of DNA from cell pellets was performed using DNeasy Blood and Tissues kit (cat # 69506, Qiagen, Germantown, MD) followed by PCR amplification of HBG locus. Sanger sequencing was carried out on the PCR product followed by TIDE (Tracking of Indels by DEcomposition) analysis (TIDE, Netherlands Cancer Institute, Netherlands) to determine editing frequency.

Flow cytometry analysis and cell sorting

Flow cytometric analysis and sorting of cell lines and human CD34+ cells were performed using the fluorochrome-conjugated antibodies listed in Supplemental Table 2. Dead cells and debris were excluded via forward light scatter (FSC)/side light scatter (SSC) gating. Flow cytometric analysis and cell sorting were performed on a FACSymphony A5, FACSCelesta, FACS Aria IIu, and Symphony S6 (BD Biosciences, San Jose, CA). Data were acquired using FACSDiva version 6.1.3 and newer (BD Biosciences). Data analysis was performed using FlowJo version 8 and higher (BD Biosciences).

Bulk RNA-sequencing

The transcripts were quantified using kallisto (v.0.45.0)⁵³. A reference transcriptome index was built for the Rhesus samples using the *Macaca mulatta* genome assembly (Mmul_10 (GCA_003339765.3)) from Ensembl (<https://useast.ensembl.org/index.html>) using the “kallisto index” command. A reference transcriptome index was built for the human samples using the *Homo sapiens* genome assembly (GRCh38) from Ensembl. The raw fastq files were aligned to the transcriptome index using default parameters and the number of bootstrap samples was set to 100. The transcript counts from kallisto were imported into R using the tximport⁵⁴ package (v.1.28.0). Gene names were assigned to the transcript IDs using the GTF file for the *Macaca mulatta* assembly for the Rhesus samples and using the GTF file for the *Homo sapiens* assembly for the Human samples. The DESeq2 (v.1.40.2)⁵⁵ package in R was used to get the normalized transcript counts for the genes of interest.

Single-cell RNA-sequencing

Alignment: The raw fastq reads were converted into gene-expression counts using the 10x Genomics Cell Ranger software suite (v2.0.0.). The cellranger mkref pipeline was used to build a custom reference using the *Macaca mulatta* GTF (*Macaca mulatta* 10.104) file from the

Ensembl database. The 10x Genomics pre-built reference for Homo sapiens was used as the reference transcriptome for the human dataset. The cellranger count pipeline was used to align the reads to the reference transcriptome with default parameters.

Dimensional Reduction and Clustering: The analysis was performed using Seurat⁵⁶ (v 4.0.2), an R package for single-cell RNAseq data. The RNA count matrix for each of the datasets was loaded in RStudio. The pre-processing encompassed the selection and filtration of cells based on the QC metrics like no. of features and percentage of mitochondrial genes and ribosomal proteins. The data was then normalized and scaled using default parameters. To reduce the effects of cell cycle heterogeneity in the data, cell-cycle phase scores were calculated based on cell-cycle markers from Rhesus and were regressed out from the data. PCA was run on the highly variable genes to compute linear dimensional reduction.

Cell type module: To determine the cell type of the cells in the data, a feature list was prepared with 3-4 specific gene markers for Stem, Erythroid, Myeloid and Lymphoid cell types for human and rhesus respectively (supplemental table 3 and supplemental table 4). A custom R script was used to assign a cell type to the cells based on the expression of the gene markers.

SPRING plots: A custom script was used to calculate the force-directed shared-nearest neighbors⁵⁷, and a dimensional reduction plot (DimPlot) was used to plot the clusters in the form of force-directed shared-nearest neighbors. A DimPlot was used to visualize the clusters grouped by their cell type. Feature Plots were made to visualize the expression of the marker genes for each of the Stem, Erythroid, Lymphoid and Myeloid clusters.

scFv Derivation from 5E10 Hybridoma

The 5E10 hybridoma cell line was used to obtain mRNA for 5' rapid amplification of cDNA ends (RACE) cloning of antibody sequences with isotype-specific primers²¹. Antibody variable regions were sequenced using an Applied Biosystems 3730xl DNA Analyzers (Thermo Fisher

Scientific). Protein sequences were reverse translated using human codons for cloning into scFv binder.

Crystallography of CD90 FAB

Fabs from 5E10 IgG were produced by papain cleavage and purified by MabSelect protein A affinity chromatography. The extra cellular domain of CD90 was expressed in HEK293 cells using the Daedalus protein expression system. A complex of 5E10 Fab/CD90 ecto-domain was isolated by SEC in crystallization buffer (12 mM PIPES ph 7.2, 150 mM NaCL, 1 mM EDTA) and concentrated to ~ 10 mg/ml. Crystals were obtained at 4°C by vapor diffusion over wells containing 100 mM phosphate-citrate (4.2), 300 mM Li₂SO₄, 0.7% 1-butanol, 18% Peg1000. These crystals were improved with the addition of 0.7% 1-butanol and several rounds of macro-seeding into drops of 1 ul of 4-8 mg/ml protein solution mixed with 1 ul of well solution. Selected crystals were transferred to a cryo-solution composed of the components of the reservoir with the addition of 15-20% glycerol, and diffraction data were collected at the Advanced Light Source (Berkeley, CA) beamline 5.0.1 and integrated and scaled with Mosflm to 1.84 Å resolution.

Initial phases were determined by molecular replacement using Phaser as implemented in the CCP4 software suite, using coordinate set 1FSK.pdb as a model of the Fab. From the initial maps it was clear that the crystal contained only the Fab. Using the amino acid sequence determined from sequencing the hybridoma, the residues that composed the complete heavy chain could be assigned and built into the electron density, however the hybridoma determined sequence of the light chain was incompatible with the electron density. Because the resolution of the diffraction data was fairly high, the light chain was initially rebuilt to have the correct number of residues in CDRs and then the identification of each sidechain was assessed and substituted with the amino acid that best fit the electron density. In the cases of structural isomers, such as glutamate and glutamine, the sequence assignment was ambiguous, but when

possible was assigned the most common structural isomer identified from sequence alignments. (This was ultimately resolved by resequencing the hybridoma.)

Iterative rounds of alternating positional refinement and model building, using the programs Refmac and COOT, including placing ordered solvent molecules, were followed by a final round of TLS refinement. Residues or side-chains that did not exhibit 2Fobs-Fcalc electron density when contoured at 0.7σ were removed or truncated to the C β atom. The quality of the final model was assessed using Molprobit.

Statistical Analysis

Statistical analysis of data was performed using GraphPad Prism version 10. Significance analyses were performed with the unpaired, two-sided Student's t-test, two-way ANOVA, or paired two-sided Student's t-test as indicated in figure legend.

Chapter 3: Combinatorial Selection Strategies Improve Gene Modification in vivo

3.1 Introduction

While HSCT remains the standard of care for treating non-malignant hematologic disorders, it requires prolonged hospitalization and significant biomedical infrastructure, limiting its accessibility to many worldwide [58, 74]. Developing injectable in vivo gene therapy delivery platforms could remove the need for HSCT in the non-malignant setting. However, current in vivo platforms in preclinical models have performed with widely variable and limited gene modification efficiencies on human HSCs [58]. As such, post-gene therapy enrichment strategies are utilized to improve the chimerism of gene-modified HSCs in vivo. Such methods include the use of positive selection platforms that give gene-modified cells a growth advantage by the expression of transgenes (i.e, truncated HMGA2 (t-HMGA2) or truncated EPO receptor (tEPOR)) [70, 75]. Others have used negative selection platforms, which remove unmodified cells using antibody drug conjugates (ADCs), chimeric antigen receptor T-cells (CAR-T cells), or chemotherapy [48, 72, 73, 76]. The concurrent utilization of both strategies has not been reported.

Here we report the targeted co-delivery of recombinant editors and transcription factors enabling a novel post-gene-therapy enrichment strategy for HSCs in vivo, without using permanent transgenes or cytotoxic agents. We have termed these particles multiplexed viral-like particles (MVPs) due to their capacity to modularly deliver transgenes, genetic editors, and signal modifiers such as transcription factors to target cells. MVPs loaded with constitutively active STAT5A protein (MVP-STAT5) increase the abundance of gene-modified HSCs after in vivo gene editing. Utilizing STAT5A-mediated enrichment and previously established

chemoselection further enhances the abundance of gene-modified cells in humanized mice. Finally, we employed this dual selection and genetic modification platform to produce HIV-resistant HSPCs via the in vivo genetic knockout of the CCR5 locus and the expression of the decoy receptor eCD4-Ig.

3.2 Results

3.2.1 Derivation of Transcription Factor-loaded MVP

MVPs are a modular viral particle platform capable of delivering a transgene and recombinant proteins, including transcription factors and Cas9-derived editors (Figure 1A). To enable HSC-targeted viral particles, producer cells were transfected with plasmids expressing knockout coccal glycoprotein (Cocal-KO), Gag-pol-rev helper plasmids, anti-CD90scfv binder, and a transgene, with or without gag-tethered-Cas9 and/or gag-tethered-STAT5A. Successful loading of all components was verified via immunoblotting of viral supernatant preps (Figure 1B). Furthermore, adding gag-tethered-STAT5A to the viral prep manufacturing did not alter the total particle number or functionally loaded titer of viral preps as measured by previously reported nanoparticle titration analysis (NTA) methodology (Figure 1C). To confirm that MVPs could deliver recombinant STAT5A to HSPCs, adult CD34⁺ HSPCs were treated with MVPs loaded with gag-tethered-STAT5A (MVP-STAT5) for 2 hrs and analyzed by immunofluorescence for recombinant STAT5A delivery by staining with anti-FLAG antibody. We confirmed both cytoplasmic and nuclear localization of STAT5A delivery after treatment with MVP-STAT5 (Figure 1D). We next sought to verify the functionality of both Cas9 editors and STAT5A protein in HSPC after particle delivery.

HSPCs from three healthy donors were treated with CD90-targeted MVPs loaded with a gRNA targeting the Beta-2-microglobulin (B2M-gRNA) locus, mNeonGreen fluorophore transgene, recombinant Cas9, with or without recombinant STAT5A in the absence of cytokines

for 12 hrs. HSPCs were then either collected for bulk-RNA sequencing or cultured for an additional five days with cytokines and then subsequently analyzed for indels at the targeted B2M locus. Both MVPs loaded with or without STAT5A could edit the B2M locus in HSPCs at an average allele editing efficiency of 17.6% and 22.5%, respectively (Figure 1E). Bulk RNA-sequencing and subsequent gene set enrichment analysis revealed that the transcriptome of HSPCs treated with MVP-STAT5 was enriched for gene sets attributed to mitotic spindle assembly, and downregulated transcripts correlating with reactive oxygen species pathways and DNA repair pathway responses (Figure 1F).

In summary, MVPs can deliver recombinant editors and transcription factors to HSPCs, leading to concomitant genomic editing and global transcriptional changes in vitro. Based on these findings, we hypothesized that MVP-STAT5 could induce robust gene modification of HSPCs and cellular proliferation while mitigating the known genotoxic effects of lentiviral and Cas9 gene modification.

3.2.2 STAT5 Positive Enrichment Improves Gene Marking Chimerism

To test the impact of the MVP-STAT5 enrichment and gene modification strategy compared to MVPs loaded with only Cas9 protein, humanized mice were intravenously injected with either particle loaded with B2M-gRNA and mNeonGreen transgene (Figure 2A). Two weeks after injection, mice were euthanized, and their bone marrow (BM) was analyzed for fluorophore and B2M expression. Finally, CD34+CD38- multipotent progenitors were FACS-sorted into colony-forming media, and genomic DNA from single colonies was analyzed for B2M locus editing.

Mice treated with either MVPs or MVPs-STAT5 displayed robust mNeonGreen positivity and ablation of B2M surface expression in human CD45+ cells as measured by flow cytometry (Figure 2B). However, mice injected with MVPs-STAT5 displayed significantly higher concomitant gene modification chimerism as measured by the expression of mNeonGreen and the lack of B2M surface expression in both hCD45+ cells (11.2 % vs. 20.2 %; $p=0.0232$) and

CD90+ HSCs (1.2 % vs. 6.5 %; $p=.0318$) (Figure 2C). This trend was reproducible in both total CD34+ HSPCs (1.7 % vs. 4.8 %) and CD34+CD38- MPPs (2.6 % vs. 5.4 %), though it did not reach significance. Analysis of CFCs from mice injected with either particle showed no significant difference in the total number of colonies formed or the distribution of colony type (Figure 2D). Finally, single colony sequencing was performed to analyze the distribution of monoallelic and biallelic editing of MPPs in either condition. We observed robust editing efficiency in colonies derived from MPPs in either treated group (37.5% vs 31.4%), though a slightly increased abundance of biallelic edited colonies in MVP-STAT5 treated mice (16.6% vs 20%) (Figure 2E). Thus, MVPs-STAT5 shows an improved capacity to enable dual transgene expression and CRISPR editing in vivo without any observed adverse effects on differentiation potential.

3.2.3 Combinatorial Positive and Negative Selection Improves Modified Cell Chimerism Compared to Single Modality Enrichment

To evaluate the benefit of STAT5A for in vivo transgene delivery, humanized mice were injected with MVP (Group 1) and MVP-STAT5 (Group 3) particles (**Fig. 3A**), and expression of mCherry was measured in the blood over time (**Fig. 3B and C**) and in the BM stem cell compartment at necropsy (**Fig. 3D**). Consistent with our prior observations (see Fig. 2), low-frequency mCherry signal was seen in the PB of mice receiving MVPs (Group 1, solid black line). However, a 2 to 3-fold increase in signal was seen when we co-delivered STAT5A (Group 3, solid red line) (**Fig. 3B**). Most importantly, we saw on average up to 16% transduction efficiency in CD34⁺CD90⁺ HSCs in mice receiving MVP-STAT5 in contrast to only 3-6% with our regular MVP (**Figs. 2E, Fig. 3D**).

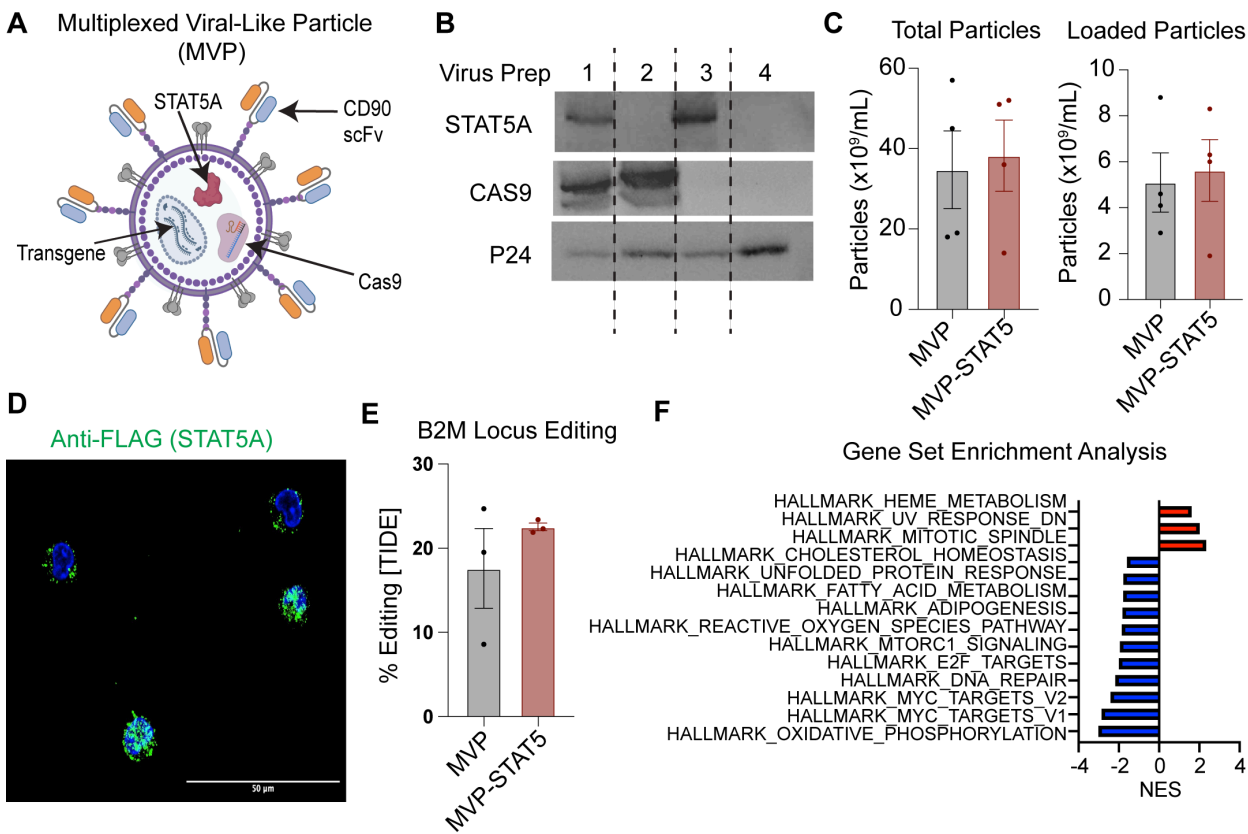
To further enrich in vivo transduced cells and confirm successful delivery of the transgene cargo, MVPs were equipped with the MGMT(P140K) chemoresistance gene,^[77] and a subset of mice was treated with MVP and MVP-STAT5 exposed to two rounds of O6BG/BCNU-treatment (Groups 2 and 4, **Fig. 3A**). Longitudinal flow-cytometric assessment of

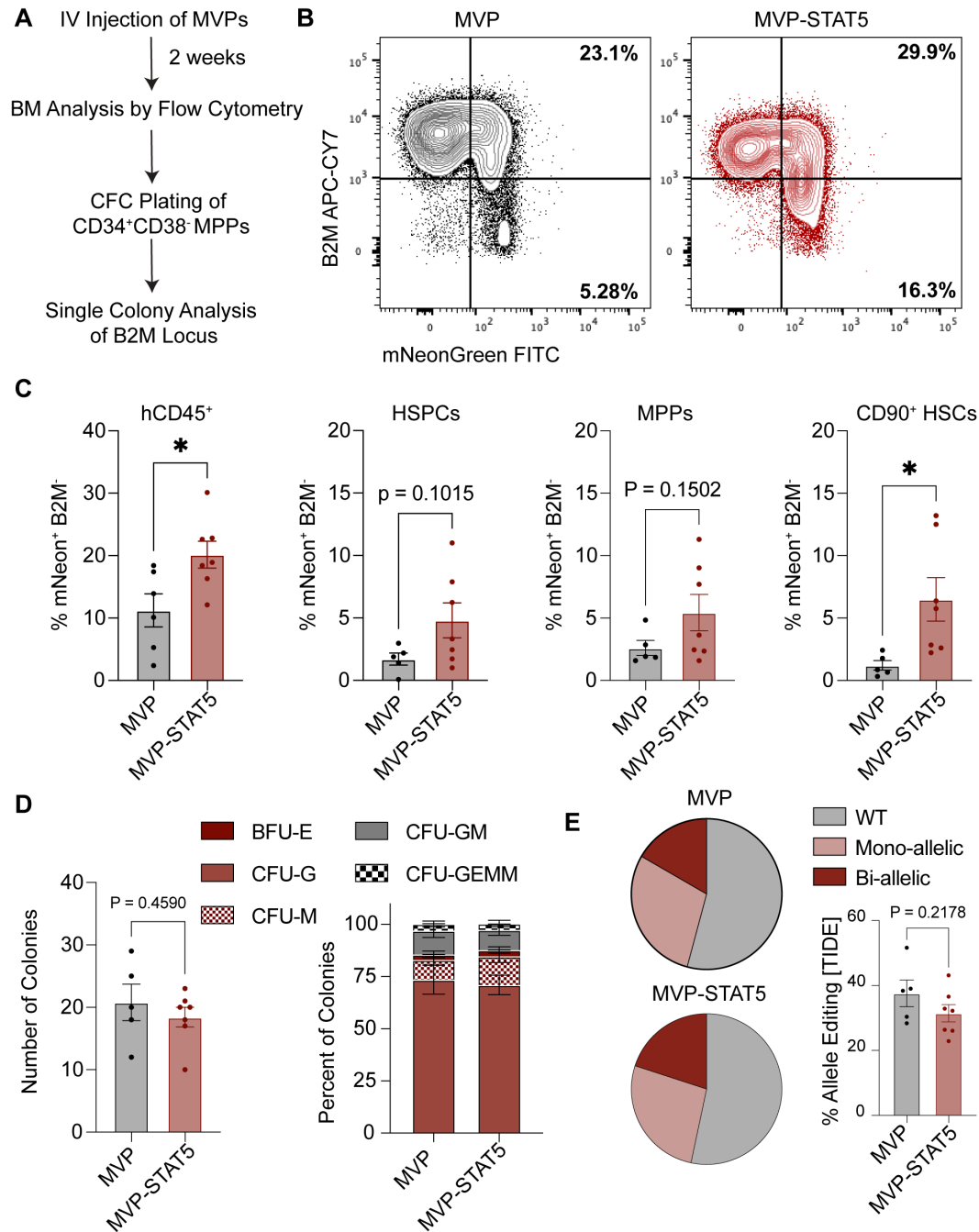
the PB showed stepwise enrichment of mCherry-expressing human CD45⁺ WBCs (**Fig. 3B**) reaching on average up to 18% in the MVP group and 30% in the MVP-STAT5 group at necropsy, with mCherry-expressing cells found in lymphoid and myeloid cells (**Fig. 3C**). Most importantly, significant enrichment of mCherry⁺ human CD45⁺ WBCs and phenotypically defined human HSPC subsets was found in MVP-STAT5A-treated mice, with CD34⁺CD90⁺ HSCs showing on average 86% transgene expression (**Figs. 3D and 3E**).

To ensure that *in vivo* transduction and chemo selection have no negative impact on clonal diversity, neonatal mice for this experiment were humanized with barcoded CD34⁺ cells using a previously described expressed barcode system.[77, 78] At the end of study, mCherry⁺ human WBCs from the BM of all four groups were FACS-purified, analyzed using 10X scRNA sequencing, and data analyzed as previously described to determine the transcriptional identity of the cells as well as identify expressed barcodes.[78] Mapping transcriptional data onto the Azimuth reference showed similar composition and identity of mCherry⁺ cells across all four groups without any obvious impact on the composition after chemo selection (**Fig 3F**). Most importantly, quantification of unique clones showed high diversity in all groups, with hundreds of barcodes detected (**Fig 3G**). Of note, the clone sizes and distribution across all four conditions closely matched our prior observations in autologous *ex vivo* gene therapy applications suggesting a stochastic contribution of *in vivo* gene-modified HSCs to steady state hematopoiesis.[79] Observed similarities in the composition, polyclonality, and clone size of mCherry⁺, chemo-protected cells in all four groups highlights that STAT5 introduction does not alter *in vivo* transduction capabilities of our MVPs but significantly enhances the contribution of gene-modified cells in steady state and upon enrichment.

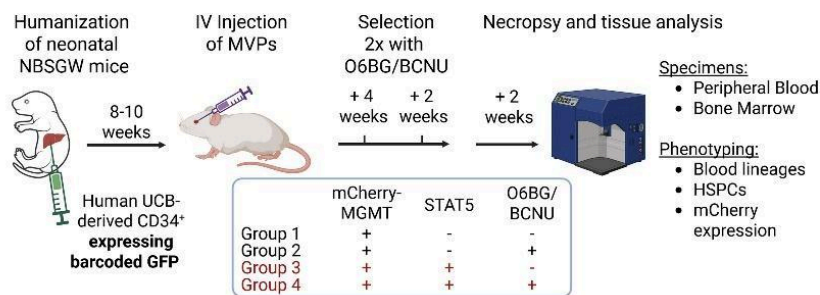
Taken together, we here demonstrate that STAT5A co-delivery significantly increases transgene delivery into human HSCs *in vivo*. In combination with chemo selection, near complete replacement of HSCs can be accomplished without impacting the clonality in hemopoietic progeny.

3.4 Figures and Legends

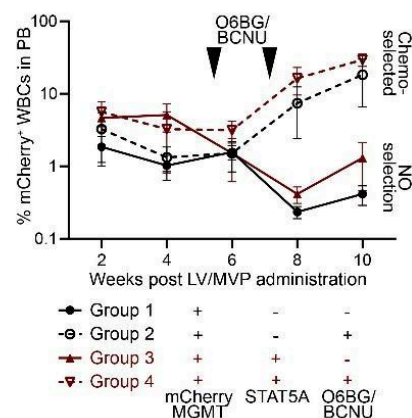




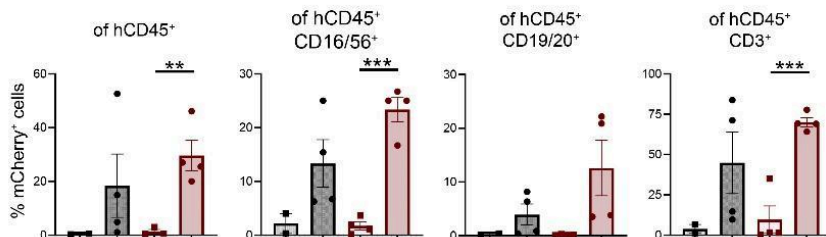
A Experimental design - STAT5A + mCherry-T2A-MGMT(P140K)



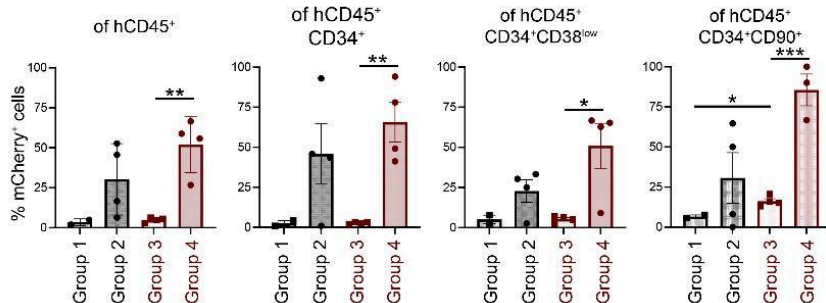
B Gene-modification and selection in PB



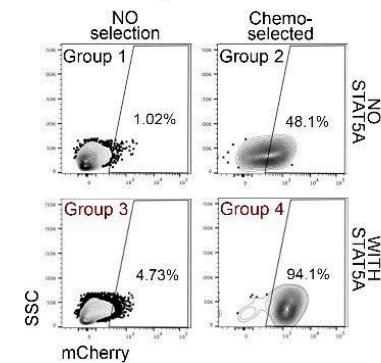
C Gene-modification and selection in PB subsets



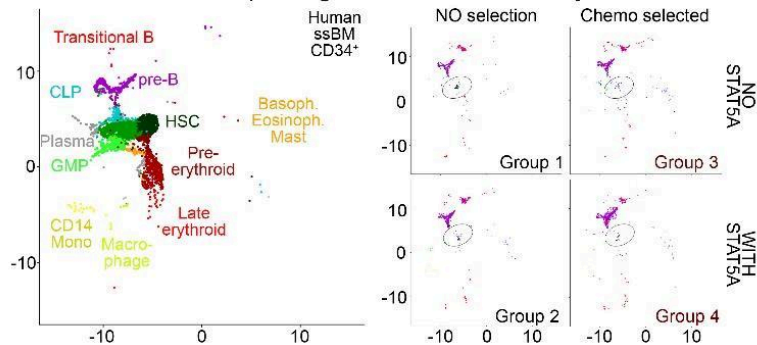
D Gene-modification and selection in the BM



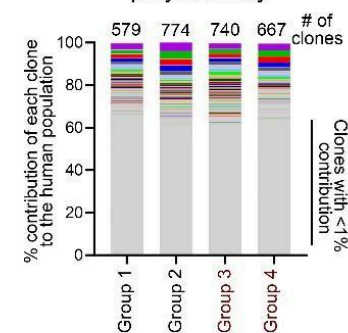
E Enrichment of mCherry+CD34+ HSPCs



F scRNAseq of engrafted human mCherry+ cells



G Maintenance of polyclonality



Chapter 4: CD90 Ablation Allows for Novel Selection/Conditioning Strategies for Gene Therapy and Hematologic Malignancies

4.1 Introduction

Hematopoiesis occurs through a hierarchical process of differentiation beginning with multipotent self-renewing hematopoietic stem cells (HSCs) that persist throughout life and ending with transient terminally differentiated unipotent cells [4, 9, 79]. The hierarchical differentiation of the hematopoietic system can be exploited in the clinic for benign and malignant hematology. A patient's pathologic hematopoietic system can be cured by replacing diseased HSCs with healthy HSCs from an HLA-matched donor (i.e., allogeneic transplantation, allo-HSCT). However, allo-HSCT is associated with significant toxicities such as graft-versus-host disease (GVHD), limiting its therapeutic potential [74]. To address this, the advent of gene modification therapies has paved the way for disease-driving mutations in a patient's HSCs to be altered by gene editing and re-transfused into patients (i.e., autologous transplantation), allowing for treatment without the significant toxicities associated with allo-HSCT. Therefore, efficient isolation, modification, and/or enrichment of HSCs has significant therapeutic potency by enabling the permanent replacement of pathologic hematopoiesis.

Established HSC isolation strategies utilize the immunophenotype pattern of CD34⁺CD45⁺CD38⁻CD90⁺/CD45RA⁻ to enrich for long-term repopulating HSCs with a prevalence of ~1 in 100 in this subset [3]. The additional concomitant expression of either EPCR or CD49f improves the prevalence of LT-HSCs to ~1 in 3 [4]. However, we and others have shown that the expression of CD90 on HSC subsets strongly correlates with increased HSC engraftment kinetics and self-renewal even within these stringently defined subsets, establishing CD90-expressing HSCs at the top of the hematopoietic hierarchy. Interestingly, CD90 is also a known marker for cancer stem cells, with high antigen expression corresponding to increased engraftment, metastasis, and chemoresistance in solid and hematologic tumors [80, 81]. Thus, anti-CD90 therapeutics would be a potent anti-cancer strategy with broad utility.

However, a limitation of such a strategy would be its detrimental impact on healthy CD90+ HSCs. As such, we aimed to elucidate whether CD90 was essential for healthy HSC functionality to further explore the potential of anti-CD90 immunotherapies for both gene therapy enrichment and cancer stem cell immunotherapy strategies. Utilizing shRNA and Base-Editing strategies, we ablated CD90 expression on human and nonhuman primate HSCs and HSC-precursor cells. We elucidated the impact of CD90 genetic modification on HSC functionality and ontogeny. We also developed an anti-CD90 chimeric antigen receptor (CD90-CAR)-T cell platform and evaluated its functionality in vitro and in vivo. These efforts led to the discovery of unique signaling regulation in developmental hematopoiesis and potent stem cell-targeted immunotherapies.

4.2 Results

4.2.1 Thy1/CD90 Knockdown has no impact on HSPC Engraftment, Differentiation, and Self-Renewal

Positive selection of CD34+ HSPCs with CD90 enriches for a hematopoietic progenitor subset with higher engraftment and self-renewal capacity. Indeed, in the nonhuman primate (NHP) model, only CD90+ cells contribute to short-term and long-term hematopoiesis after autologous stem cell transplantation [9]. However, the role of CD90 signaling in contributing to HSC identity or these engraftment phenotypes remains unknown. We therefore sought to study whether the expression of CD90 was crucial for HSC functionality.

We screened three putative shRNA sequences targeting the Thy1 loci in the CD90+ K562 immortalized cell line to develop a CD90 knockdown strategy. The Thy1-shRNA-1 sequence displayed the highest ablation capacity of the sequences screened. Thus, we designed a lentiviral transgene payload with the expression of Thy1-shRNA-1 driven by the U6 promoter and the reporter gene mCherry under Ef1 α promoter regulation; hereby referred to as CD90-KD or KD. To evaluate engraftment and differentiation of HSCs with depleted CD90, cord blood (CB) derived CD34+ HSPCs were transduced with CD90-KD, or a control scramble

shRNA (Control) and transplanted into NBSGW neonates (Figure 1A). Peripheral blood (PB) chimerism was monitored via biweekly bleeds and evaluated by flow cytometry. After 20 weeks post-transplant, bone marrow (BM) was assessed for human chimerism, lineage composition, HSPC abundance, and reporter gene expression. Finally, to evaluate the self-renewal capacity of transduced cells, CD34^{hi} HSPCs from the BM of engrafted animals were FACS-purified and intraosseously injected into one femur of busulfan-conditioned adult mice. After 12 weeks, BM was evaluated for total human chimerism and gene-reporter chimerism in secondarily transplanted mice.

Analysis of PB gene-reporter expression measured as percent mCherry positive fluorescence in hCD45⁺ cells remained stable in primary transplanted mice who received HSPCs transduced with control or CD90-KD vector. Furthermore we observed no lymphoid or myeloid biased gene marking as seen by stable expression of the reporter gene in both CD19⁺/20⁺ B cells and CD14⁺ monocytes even up to 20 weeks post-transplant (Figure 1B). Additionally, CD90-KD mCherry⁺ cells were present in both CD3⁺CD4⁺ and CD3⁺CD8⁺ T-cell lineages (Supplemental Figure). To evaluate whether CD90-KD's impact would be seen in progenitor pool engraftment, we analyzed the BM of transplanted mice at necropsy for mCherry⁺ HSPC subset abundance. We observed no difference in the abundance of CD34⁺ HSPCs or CD34⁺CD38⁻ multipotent progenitors (MPPs) in control BM compared to KD BM of transplanted mice (Figure 1C-D). However, as confirmation of our ablation strategy, we observed a 55% reduction ($p=.0318$) in total CD34⁺CD90⁺ HSCs in CD90-KD BM compared to control BM (Figure 1E). To determine whether the loss of CD90⁺ cells in KD cells was due to loss of HSCs versus intended inhibition of CD90 translation, we analyzed the abundance of CD34⁺CD38⁻CD133⁺ cells as a surrogate HSC immunophenotype. Our analysis of HSPC and MPP abundance revealed no difference in the abundance of CD133⁺ cells between KD BM and control BM (Figure 1E). Furthermore, we observed a consistent frequency of CD34⁺CD38⁻CD45RA⁻EPCR⁺ cells in both CD90-KD and control mCherry⁺ mice. BM of

secondary transplant NBSGW transplanted with CD90-KD cells displayed no engraftment deficit compared to control, and equal abundance of positive mCherry signal as primary transplants confirmed self-renewal of CD90-ablated HSCs (Figure G-I).

In summary, CD90-KD HSPCs shows no deficit in engraftment, lineage differentiation, or self-renewal compared to their wild-type counterparts. Indeed, terminally differentiated hematopoietic lineages (i.e, B-cells, T-cells, Monocytes) were found to be represented in CD90-KD mCherry+ cells. Other than CD34+CD90+ population abundance, evaluation of CD90-KD HSPC subsets also showed no depletion of relative abundance compared to control HSPCs. Finally, self-renewal of CD90-KD HSCs was not perturbed due to the loss of CD90+ expression.

4.2.2 Ectopic Modulation of CD90 Perturbs the Definitive Wave of Embryonic Hematopoiesis

Thy1/CD90 expression is highly abundant in the HSC-precursor arterialized endothelium of Carnegie Stage 15 human dorsal aorta gonad mesonephros (AGM) tissue [82]. These findings have been recapitulated utilizing in vitro hematopoietic differentiation models of human pluripotent stem cells (hPSCs). As the endothelial to hematopoietic transition (EHT) occurs, giving rise to nascent HSCs, rounded putative HSPCs lower their Thy1/CD90 expression [83-85]. Based on these findings, we sought to explore whether Thy1/CD90 expression was crucial to EHT and whether modulation of Thy1 expression could either enhance or decrease hematopoietic output.

Utilizing a previously reported monolayer hematopoietic differentiation protocol, day 5 endothelial cells (D5 ECs) or day 9 hemogenic endothelium cells (D9 HECs) were transduced with either control lentivirus, CD90-KD lentivirus, or a CD90-t2a-mCherry overexpression vector (CD90-OE) and further subsequently differentiated into hematopoietic progenitors (HPs) [83]. Differentiation of transduced cells was tracked utilizing flow cytometry and single-cell

RNA-sequencing (sc-RNA-seq) on day 9 (D9) or day 19 (D19) of the differentiation protocol, coinciding with mature endothelial arterialization and EHT, respectively (Figure 2A).

Transduction of D5 ECs with CD90-KD or CD90-OE led to significant abrogation of arterialized HEC differentiation on D9 as measured by the percentage of CD34⁺VE-Cadherin⁺CXCR4⁺DLL4⁺ cells out of all cells assessed (91.6% (Scramble) vs. 31.6% (CD90-KD) vs. 3.84% (CD90-OE)), though with disparate phenotypes (**Fig. 2B**). CD90-KD cells showed a lower VE-Cadherin (VEC) expression and drastically lower delta-like ligand 4 (DLL4) expression but maintained CD34 and CXC chemokine receptor 4 (CXCR4) expression. In contrast, cells expressing the CD90-OE cassette showed lower CD34 expression. Cells that maintained CD34 expression also showed lower CXCR4 expression, while maintaining a high expression of DLL4. To assess if hematopoietic differentiation could be spared if ectopic manipulation of CD90 was performed later in arterial specification, we transduced ECs at D9 of differentiation and assessed for hematopoietic output on D19. Compared to Scramble transduced cells, we observed a lower percentage of HPs in D19 cultures in CD90-KD (12.4% vs 4.7%) and CD90-OE (12.4% vs. 0.3%) cultures. Furthermore, CD90-OE endothelial cells displayed a lower endothelial cell-selective adhesion molecule (ESAM) expression than CD90-KD (31.6% vs 98.9%) and Scramble (31.6% vs 94.1%) cultures. To better understand the mechanism by which CD90 regulates hemogenic endothelium identity, we performed single-cell RNA-sequencing on D9-differentiated cells.

Unsupervised clustering revealed two main clusters, with cluster 1 enriched for both Scramble and CD90-KD cells and cluster 2 enriched for CD90-OE cells (**Figure 2D**). All clusters homogeneously expressed CDH5, KDR, and Runx1 confirming the maintenance of HE identity. However as seen in our flow cytometry analysis, CD90-KD and CD90-OE showed a decreased expression of DLL4 and CXCR4. CD90-OE cells also had lower expression of CD34. We next performed gene set enrichment analysis on the differentially expressed genes between each sample to better understand the pathways that were perturbed through CD90 modulation. Top

gene sets enriched were related to vascular development, cell adhesion, and VEGFR2 (KDR) signaling. As no mechanistic regulatory link between CD90 and VEGFR2 has been previously defined, we explored if CD90 modulation perturbed the cellular interaction landscape of HE via ligand-receptor analysis. Additionally, we analyzed the interactions between transduced and non-transduced cells in each condition to better elucidate whether the perturbation we observed were largely cis- or trans- regulated.

4.2.3 Base-Editing Strategy for CD90 Ablation does not perturb HSPC Function

Adenine Base editors (ABE) contain a catalytically impaired Cas protein fused to a nucleotide deaminase allowing for the conversion of A•T base pairs to G•C. The Cas9 domain directs the DNA-modifying enzyme to directly convert one base to another at a guide RNA-programmed target site allowing for high allele specificity of genomic editing. Nearly half of pathogenic point mutations can be corrected by converting an A•T base pair to a G•C base pair, a strategy utilized in clinical trials for the disease backgrounds of Sickle Cell Disease (SCD) and polycystic kidney disease (PKD). Furthermore, ABE has been shown to have broad capacity to act as a genetic knockout tool by the point mutagenesis of start codon sites (i.e., ATG sequence). We sought to develop an ABE genomic editing strategy to ablate CD90 in HSCs that could potentially be multiplexed with other therapeutic gene engineering approaches.

We utilized the CRISPR RGEN Tool BE-Designer to develop gRNAs targeting the start codon of the Thy1 allele utilizing ABE8E-NG BE enzyme. Two gRNAs were screened for their capacity to target the Thy1 start codon, with one, referred to as CD90BE-g2, showing 50% editing in HEK293T cell line after electroporation with ABE8E mRNA and CD90BE-g2 (CD90BE) (Figure 3A, Supplemental Figure). We further evaluated the feasibility of this strategy by electroporating CD90BE into human HSPCs from two independent donors. Editing analysis using EditR software showed an average of 18% editing at the Thy1 start codon (A4) with potential bystander adenine edits (A2, A3, A5, A6) occurring at an average of 4% editing and 2% editing at A2, A3, and A5, A6 sites respectively (Figure 3B).

We next sought to confirm maintenance of HSPC functionality after CD90-BE treatment. Adult mobilized peripheral blood CD34⁺ HSPCs were treated with CD90-BE and transplanted into busulfan conditioned NBSGW mice. Twenty weeks post-transplant, bone marrow (BM) was evaluated for human engraftment, lineage distribution, HSPC abundance, and allele editing frequency. We observed equal engraftment of control HSPCs or CD90-BE HSPCs in NBSGW mice, as shown by the percentage of positive hCD45⁺, hCD34⁺, and hCD34⁺CD38⁻ cells in the population (Figure 3C-E). Like CD90-KD, we observed a 22% decrease in CD34⁺CD90⁺ cells ($p=.048$) in CD90-BE HSCs though with a 7.3% increase in CD133⁺ cells ($p=.0344$) (Figure 3F-G). Additionally, we observed the presence of persistent Thy1 allele editing in the BM at an average of ~10% comparable to the average editing efficiency of the infusion product (12%) on day zero of transplant (Figure 3H). Further optimization of CD90-BE protocol was performed on both human and NHP cells to assess its potential as a therapeutic editing platform, conferring up to 80% base editing in immortalized and primary cells (Supplemental Figure).

We have previously reported the strong correlation of the number of CD90⁺ HSCs in the transplant infusion product and the time of hematopoietic recovery of white blood cell (WBC), platelet, and neutrophil count after autologous transplantation in NHP. Historically, NHP transplants with infusion products of less than 150,000 CD90⁺ cells/kg led to engraftment failure within the first 30 days. Based on our human engraftment studies on CD90 ablation, we sought to perform a proof-of-concept study in the NHP model focusing on the safety of simultaneous ablation of CD90 and therapeutic genomic editing in HSCs. A Rhesus Macaque was mobilized with GCSF and Plerixafor and peripheral blood stem cells were collected via apheresis and CD34⁺ enrichment. After cell enrichment, NHP HSPCs were cultured for 24 hours and electroporated with ABE8E mRNA, CD90-gRNA, and the HBG-175 gRNA, previously reported to induce expression of fetal hemoglobin and rescue pathologic sickling in SCD. The study animal was conditioned for five days with busulfan (5 mg/kg) and transfused with genetically engineered HSPCs. Engraftment and gene editing chimerism was tracked through daily cell

blood counts (CBCs), flow cytometry of regular peripheral blood draws, and Sanger sequencing of the Thy1 and -175 HBG loci (Figure 4A).

Successful multiplex editing of transplant infusion product was confirmed by Sanger sequence of Thy1 and -175 HBG loci followed by EditR software analysis. Thy1 allele analysis showed an 84% base editing efficiency at the A4 site. Analysis at the HBG site also confirmed an editing efficiency of 84%. Compared to historical busulfan conditioned rhesus macaque transplants, we observed no deficit in WBC, platelet, or neutrophil recovery. WBC recovery in CD90-KO animal occurred on day 12 post-transplant compared to the average 13.8 ± 8.5 days in historical transplants. Platelet recovery in CD90-KO animal occurred on day 22 post-transplant compared to the average 41.6 ± 18.0 days in historical transplants. Neutrophil recovery in CD90-KO animal occurred on day 12 post-transplant compared to the average 15.0 ± 9.3 days in historical transplants (Figure 4B). Sanger sequencing and EditR analysis confirmed gene edited chimerism as early as 3 days post-transplant at 4% editing efficiency with steady increase over time reaching a maximum of 77% Thy1 allele editing by day 30 post-transplant (Figure 4C). Increase in edited allele chimerism was concordant with decreased expression of CD90 on the cell surface of first granulocytes and then natural killer cells as measured by flow cytometry analysis; reaching minimums of 22% and 3% CD90+ cells respectively at end of study (Figure 4D). Analysis of HBG allele editing showed parallel engraftment chimerism with early detection of 3% edited alleles on day 3 post-transplant and a maximal allele editing chimerism of 67% at d14 post-transplant followed by plateau (Figure 4E). Additionally, fetal HbF expression was confirmed by flow cytometry of peripheral red blood cells, reaching up to 40% HbF+ RBCs (Supplemental Figure).

BM analysis of transplanted CD90-KO NHP showed CD90+CD45RA- cells representing only 2.78% of CD34+ cells in contrast to pretransplant BM analysis showing CD90+CD45RA- cells represented 22.1% of CD34+ cells (Figure 4F). We FACS-sorted total CD34+ HSPCs, CD34+CD90+CD45RA- progenitors, CD34+CD90-CD45RA- progenitors,

CD34+CD90-CD45RA+ progenitors, B-cell lymphocytes, T-cell lymphocytes, granulocytes, monocytes, and natural killer cells from BM collection and performed MiSeq Next generation sequencing (NGS) analysis to measure both Thy1 and HBG editing frequency. DNA sequencing analysis revealed stable and pan-contribution of cells with edited alleles amongst HSPCs subsets and terminal lineages analyzed (Figure 4G). Furthermore, primary CFCs from CD34+ HSPCs, CD34+CD90+CD45RA- progenitors, CD34+CD90-CD45RA- progenitors showed multilineage differentiation capacity (Supplemental Figure).

In summary, ablation of CD90 surface expression can be accomplished by employing ABE8E directed towards the ATG codon start site of the Thy1 allele. CD90 ablation did not impact HSC engraftment in NBSGW xenograft models and NHP autologous BM transplants.

4.2.3 In Vitro Validation of a CD90-directed CAR-T Cell

With our ability to ablate CD90 in adult HSCs without deleterious impact on engraftment or multilineage differentiation potential, we next devised a means to eliminate CD90+ cells. To this end, we installed CD90-directed short chain variable fragments (scFvs) into a 2nd generation chimeric antigen receptor (CAR) backbone with CD28 co-stimulation and a truncated EGFR (tEGFR) reporter. scFvs were generated from a previously characterized human/NHP cross-reactive anti-CD90 antibody (5E10) by varying the order of the heavy chain (VH) and light chain (VL) as well as using two different linkers, G4S or whitlow. Successful transduction of T cells was confirmed by FACS analysis of tEGFR, demonstrating no significant differences between different CD90 CAR (CAR90) constructs. Cytotoxic function of CAR90 T cells was first vetted in vitro by Incucyte, demonstrating dose-dependent killing of CD90+, but not CD90-, 293 T cells relative to untransduced T cells. Assessment of secondary markers of activation demonstrated that CAR90 T cells co-cultured with CD90+ cells upregulated the T-cell activation marker CD25 and produced the pro-inflammatory cytokines IL-2, TNFa, and IFN- γ . To confirm the functionality of CAR90 in a biologically relevant cancer model, we co-cultured CAR90 T cells with the T-cell acute lymphocytic leukemia (T-ALL) Jurkat cell line. Evaluation of total cell count

by FACS demonstrated complete ablation of Jurkat cells after 48h of co-culture with CAR90 T-cells.

Having demonstrated the functionality and specificity of CAR90 validated in cell lines, we next wanted to determine the impact on primary HSCs which express significantly less CD90. To this end, we cultured CAR90 T cells with HSPCs for 48h before immunophenotyping by FACS as well as plating into primary and secondary colony forming assays. Co-culture of HSPCs with UTD T cells led to a partial decrease of CD90, however co-culture with CAR90 T cells led to a significant further reduction of CD90 positivity. As in our experiments with cell lines, T-cell activation was secondarily confirmed by assessing CD25 and production of pro-inflammatory cytokines. Further, while HSPCs from both UTD and CAR90 co-culture were able to give rise to multilineage colonies in primary CFC, CAR90 entirely ablated secondary colony forming potential, consistent with loss of the HSC population. With the ability of CAR90 to specifically eliminate CD90+ cells, both cancerous and non-edited HSCs, we turned towards their application in vivo.

4.2.4 CD90 editing protects modified HSPCs from CAR90 T-cells in vivo

To evaluate whether CD90 ablation confers protection from CAR90 T cells, NBSGW mice were engrafted with human HSPCs edited by CD90BE. Additionally, to assess whether CD90 modification could also act as an enrichment handle for therapeutic purposes, HSPCs were concomitantly edited at the HBG-175 site with ABE8E. Mice were then treated with CAR90.1 (hereafter referred to as CAR90) T cells and monitored for 14 days before necropsy and collection of HSPCs for FACS analysis and colony forming assays. CAR90 T cells expanded in vivo, leading to a peak CAR marking observed 7 days post-treatment (DPT) in the peripheral blood (21.2%).

At necropsy, we observed treatment with CAR90 did not significantly impact the total number of hCD45+ cells. However, CAR90 did lead to significant decreases in CD90+ HSCs (1% vs. 3%),

MPPS (0.25% vs. 1%), and total HSPCs (1.75% vs. 0.75%). To confirm the maintenance of the self-renewing HSC population and the selective advantage of CD90 modified cells, CD34+CD133+CD38-CD45RA- HSPCs were plated into primary and secondary colony forming assays. CAR90 did not significantly impact colony forming potential nor skew HSPC differentiation in both primary and secondary cultures. However, assessment of editing revealed partial enrichment of CD90 edited cells in primary culture of CAR90 treated animals compared to UTD treated mice, and a significant 2.8 fold enrichment of CD90 edited alleles in secondary culture. Finally, we evaluated whether enrichment of CD90 edited cells corresponded to a concomitant increase in the multiplexed HBG -175 edited alleles. We observed a concomitant increase in base-editing HBG-175 in CAR90 treated mice compared to UTD mice. Thus, CD90 ablation via base editing can be utilized as a gene-enrichment handle for increasing the gene modification chimerism of therapeutic gene edits.

4.2.5 CD90 editing of HSCs enables a leukemia treatment strategy

With the safety of CAR90 demonstrated *in vivo*, we set out to evaluate the ability of CAR90 to rescue a human leukemia xenograft model. NBSGW mice were engrafted with CD90 ablated human HSPCs for 6 weeks. The leukemia Jurkat cell line was then introduced, followed four days later by either 2×10^6 UTD or CAR90 T cells. Mice were then monitored for Jurkat outgrowth biweekly by bioluminescent imaging (BLI) and by semiweekly blood draws. Jurkat outgrowth was observed in the UTD condition while CAR90 treated mice controlled the tumor starting at 8 DPT. This difference observed in BLI corresponded with a significantly increased mean survival (15D vs. >30D). CAR90 treated mice demonstrated rapid outgrowth of T cells one-week post-treatment, a trend which continued to three-weeks post-treatment. This corresponded with a concomitant increase in CAR marking. At necropsy, we found that there were no significant impacts of CAR90 treatment compared to UTD treatment across most hematologic populations (total CD45s, B-cells, monocytes, HSPCs, & MPPs). The lone

exceptions were T cells, which were significantly increased in the CAR treatment group, and CD90+ HSCs, which were nearly totally ablated in the CAR90 treated animals.

To confirm that CD90 ablated HSCs were protected from CAR90, even with the Jurkat cells acting as a significant CD90 antigen source, we harvested HSPCs and plated them for primary and secondary colony forming assays. We did not observe any impact on multilineage differentiation potential in either primary or secondary colony forming assays. We similarly observed enrichment for CD90 edited cells in CFC cultures of UTD treated mice compared to CAR90 treated mice; as well as an increase in editing in secondary cultures of CAR90 treated mice compared to primary culture.

In summary, CAR90 is well tolerated and effective in eliminating CD90+ leukemia cells *in vivo*. CD90 ablation of HSCs confers specific protection to our CAR90 T cells, preserving hematopoietic integrity while the leukemia cells are selectively targeted.

4.3 Discussion

Herein, we report on our work characterizing the function of CD90. Through transduction with a CD90-directed shRNA we demonstrated its dispensability, permitting complete, multilineage hematopoiesis in a xenograft mouse model with no adverse impacts to primary nor secondary engraftment. We further investigated the role of CD90 in early hematopoiesis through KD and OE in an *in vitro* model of hematogenesis from the arterial hemogenic endothelium, demonstrating its core function as a mediator of neonatal hematopoiesis. We then sought to evaluate the utility of CD90 knockout and targeting for therapeutic modalities. To this end, we created a clinically relevant ABE approach to knockout CD90 and validated its efficacy and safety in murine and NHP models. Simultaneously, we developed and screened *ex vivo* CAR90, demonstrating specific, CD90-directed CAR toxicity. Finally, we investigated the utility of CD90 KO and CAR90 in two leukemia models, demonstrating therapeutic benefits in both an engineered cell line model and primary AML samples. These studies provide critical insight into

the role of CD90 in fetal and adult hematopoiesis as well as demonstrate the utility of its targeting for the treatment of malignant LSCs.

We first investigated the role of CD90 in adult hematopoiesis utilizing shRNA-based knockdown, revealing minimal impact on HSC function. Mice treated with CD90KD did not deviate from scramble-treated controls in their total hCD45 engraftment, nor in the composition of the peripheral blood. At necropsy, we found that CD90KD successfully reduced the population of CD90+ cells in the BM but did not adversely impact several other markers of long-term HSCs, including EPCR and CD133. To ensure that CD90KD did not adversely affect long-term HSC function, we serially engrafted a 2nd cohort of mice with CD90KD or scramble-transduced HSCs, revealing equal secondary engraftment potential. Taken together, these findings demonstrate the vestigial nature of CD90 in adult hematopoiesis.

Next, we sought to investigate the role of CD90 in fetal hematopoiesis. To this end, we transduced human hPSCs with CD90KD, CD90OE, or scramble control lentiviral constructs. We then directed these hPSCs through hematopoietic differentiation in vitro, revealing that both CD90KD and CD90 OE significantly reduced the potential of hPSCs to arterialize and undergo EHT successfully. To investigate the molecular mechanisms driving this finding, we carried out scRNAseq on D9 revealing CD90's role in upregulating VEGF-VEGR2 signaling through its potentiation of integrin activity.

Knowing that CD90 is necessary in early hematopoiesis but dispensable in adult hematopoiesis, we developed a clinically relevant CD90 ABE strategy to ablate CD90. We found that CD90 was amenable to ABE-mediated KO. Further, we discovered that CD90KO HSPCs engrafted in xenograft mice similarly to non-edited control cells, with no statistically significant differences in total hCD45+, CD34+, or CD34+CD38- engraftment. We found that the CD90+ population was decreased in our CD90 knockout (CD90KO) mice, as expected. To further advance this technology, we then treated two NHPs with CD90KO HSCT. We found that CD90 knockout did not adversely impact hematopoietic recovery as measured by time to platelet and

neutrophil recovery. However, following transplant we found that CD90 expression on granulocytes and NK cells dropped precipitously, in line with observed CD90 editing in the peripheral blood. Finally, we investigated the rate of CD90 editing in diverse WBC and HSPC populations, revealing comparable levels of editing across populations. Together, these data reconfirm the dispensable nature of CD90 in adult hematopoiesis and validate our ABE approach to create a CD90- population of HSCs.

With our ability to ablate CD90 without adverse impacts on hematopoiesis established, we sought to evaluate whether CD90 could be targeted by CAR T cells. To this end, we created four CAR90 constructs and demonstrated their capacity to kill CD90+ cells in vitro, produce pro-inflammatory cytokines, and upregulate T-cell activation markers. We then established that despite lower CD90 antigen density, CAR90 successfully targeted CD90+ HSCs within the HSPC compartment, reducing CD90 MFI and ablating serial replating potential.

With our ability to target CD90 established in vitro, we next looked to investigate whether our previously established CD90KO strategy would protect edited HSCs from CAR targeting. We engrafted mice with CD90KO HSPCs. Six-weeks post-transplant, mice were challenged with the CD90+ Jurkat cell line then treated with CAR90 or UTD T cells. We found that CAR90 T cells expanded in vivo and controlled Jurkat outgrowth. This finding correlated with an increase in survival in CAR90 treated mice relative to UTD control mice. We also found that CAR90 significantly reduced the population of CD90+ cells in the BM of treated mice, but did not adversely impact overall hCD45 engraftment or constituent WBC populations. We did observe a significant decrease in CD90 positivity in the HSPC compartment, suggesting that CAR90 was able to efficiently clear both CD90+ jurkat cells as well as CD90+ unedited HSCs. This finding was further supported by the increased editing observed in the BM of CAR90 treated mice relative to UTD controls.

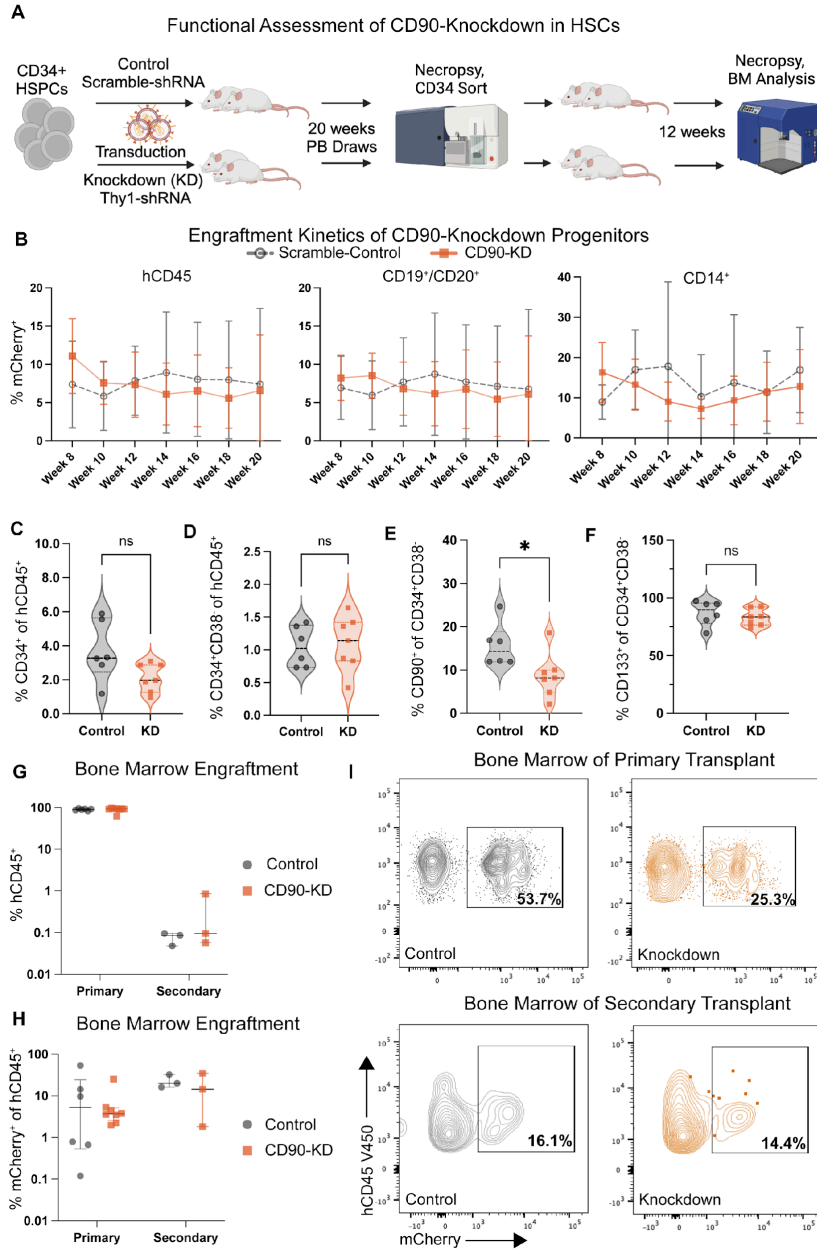
Targeting antigens shared between HSPCs and leukemia cells has been investigated by several groups, including our own [48, 73, 86, 87]. While initially trained on CD33, more recent

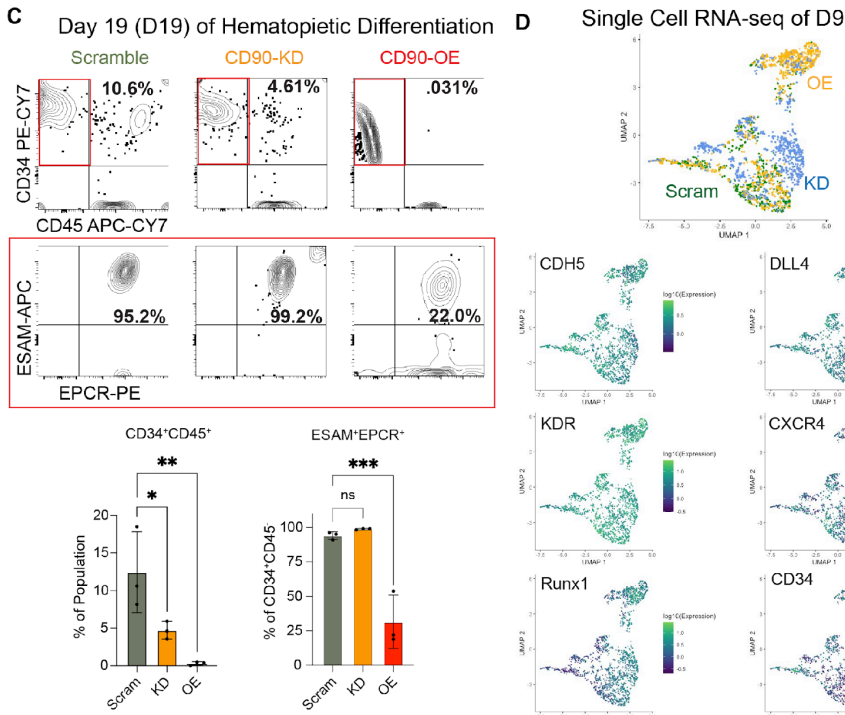
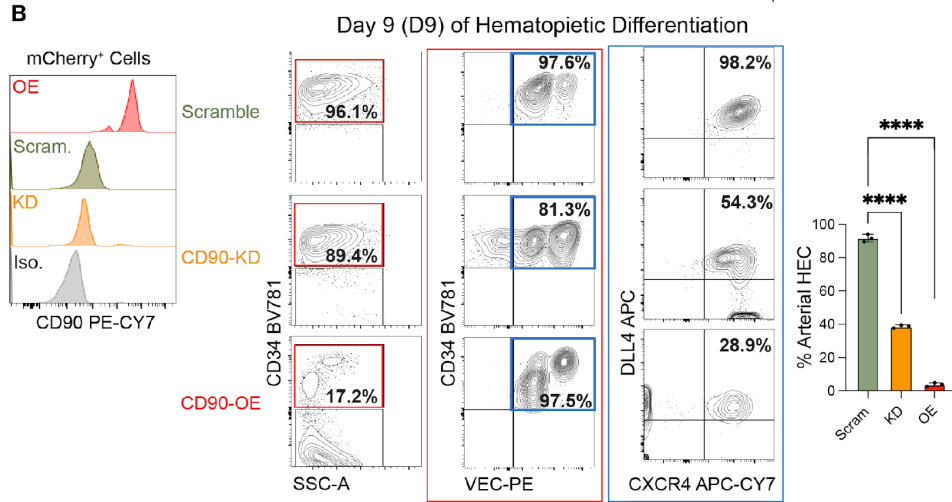
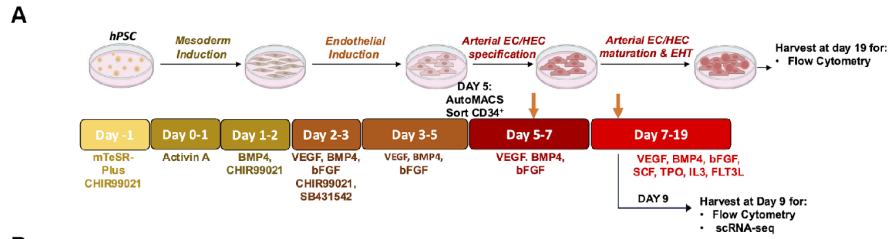
works have expanded the lens of HSPC modification with CAR-mediated targeting to include CD117, CD125, and CD45 [73]. However, stymying many of these approaches is antigen heterogeneity, with some portion of leukemic cells not expressing the target antigen, thereby evading detection & killing. Given CD90s established role in hematopoiesis as well as its previously described identification on LSCs, we are hopeful that combinatorial targeting of CD90 will allow treatment of both primary disease burden alongside the LSCs majorly responsible for leukemia relapse [81].

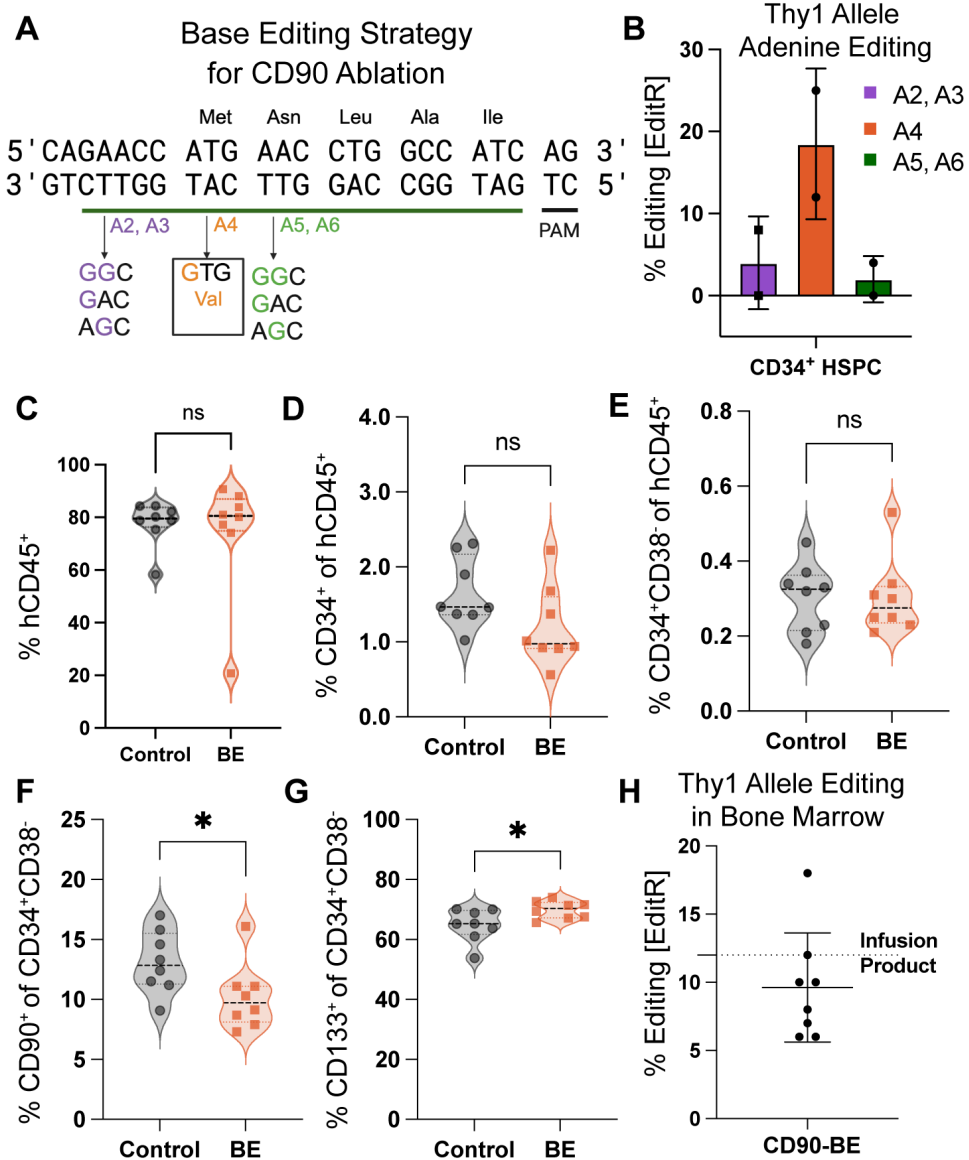
In addition to our application of CD90KO/CAR90 in the setting of oncology, we see potential for this approach as a post-transplant gene-enrichment strategy. Others have reported on a critical therapeutic threshold of allele editing to maintain therapeutic efficacy post-transplant (i.e., SCD >30%, HIV >75%) [88, 89]. However, should engraftment wane below this level, the current best recourse is serial transplant, exposing patients to HSCT associated morbidity and mortality. Our study represents one of the first reports of post-transplant gene enrichment, paving the way for a new application of CAR T cells in a non-malignant setting.

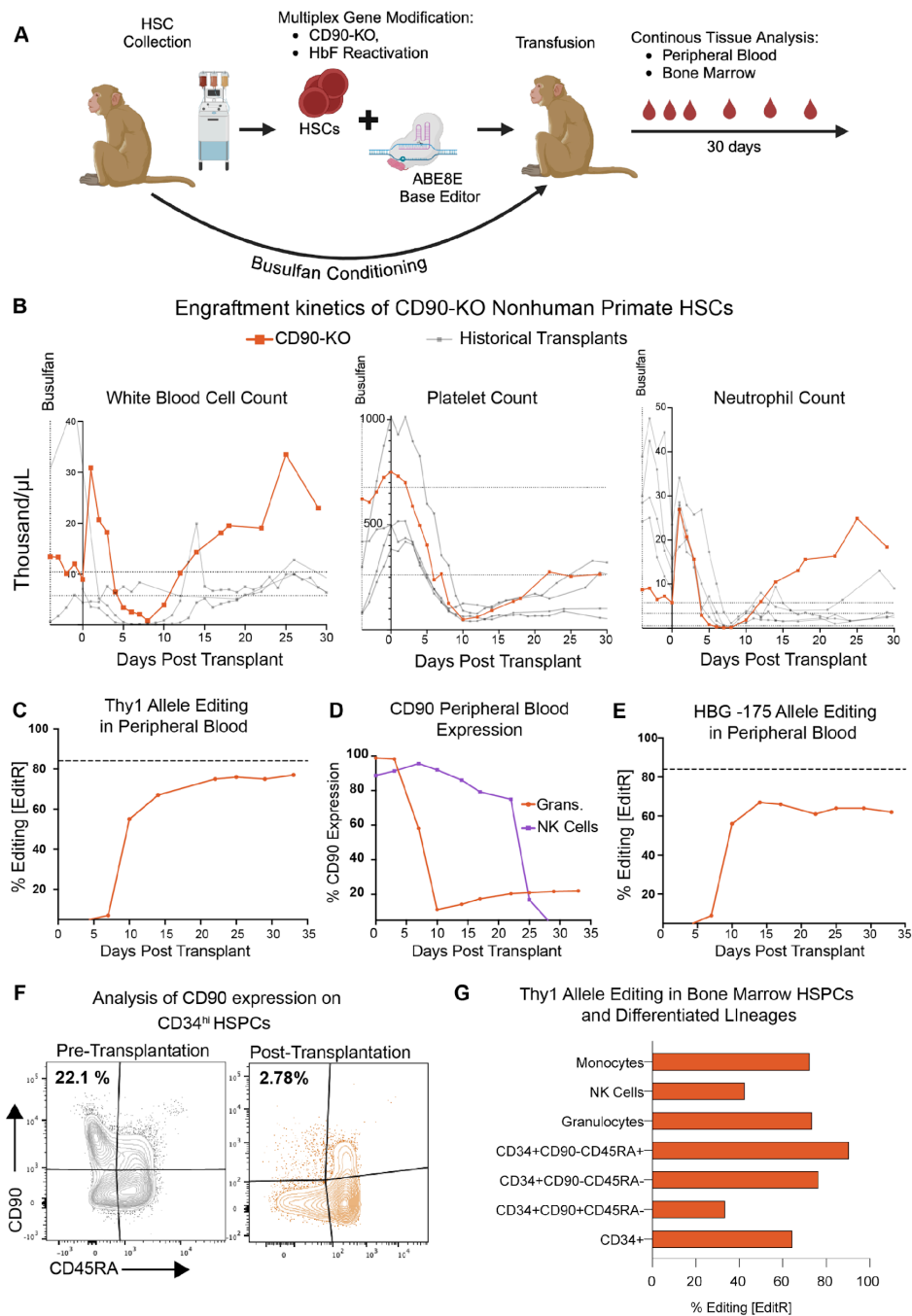
In conclusion, we have elucidated the function of CD90 in neonatal hematogenesis and demonstrated its dispensability in adult hematopoiesis. We have developed a clinically relevant system to ablate CD90 utilizing ABE and target CD90+ cells by CAR90. This combined approach liberated CD90 as a target for anti-cancer applications, allowing targeted elimination of CD90+ LSCs.

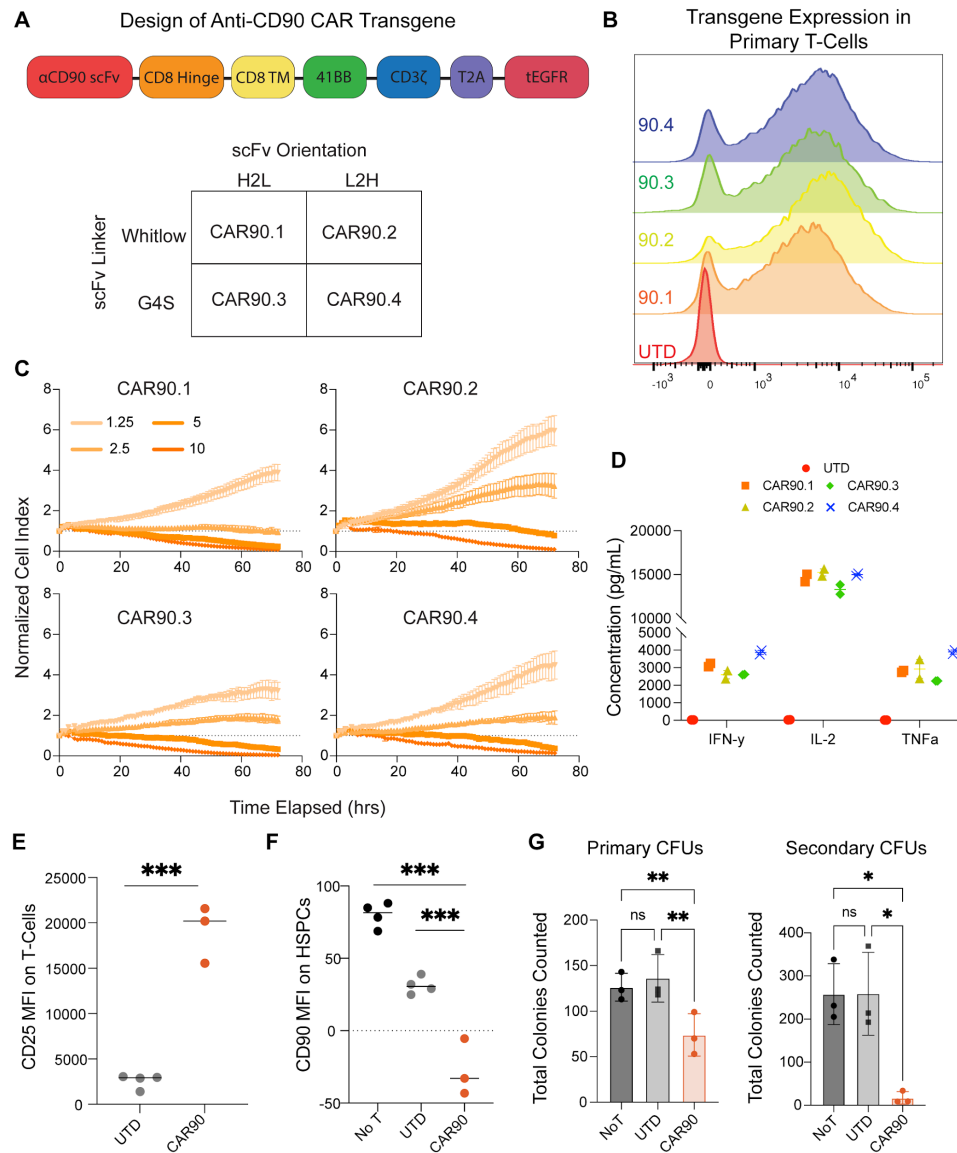
5.4 Figures and Tables

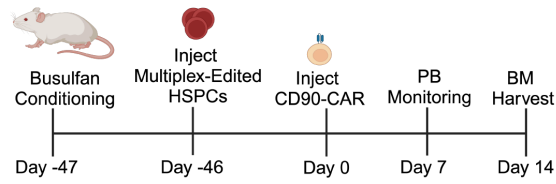
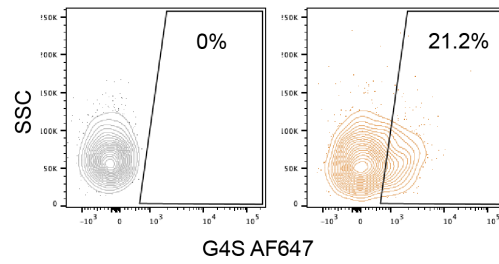
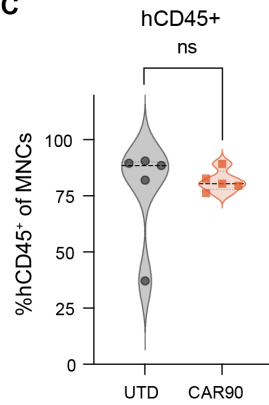
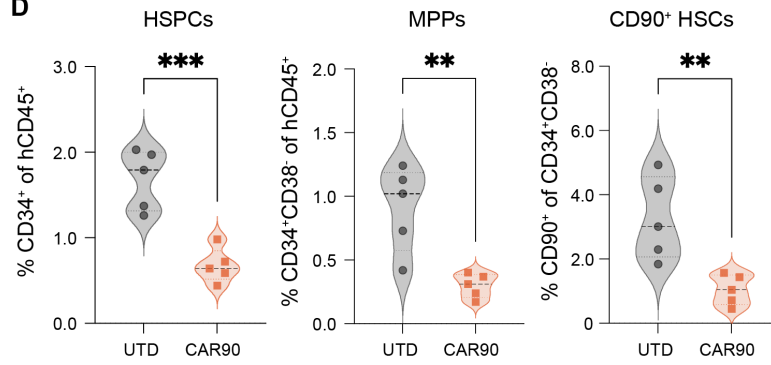
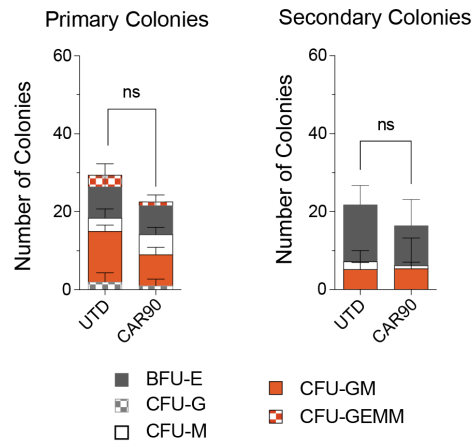
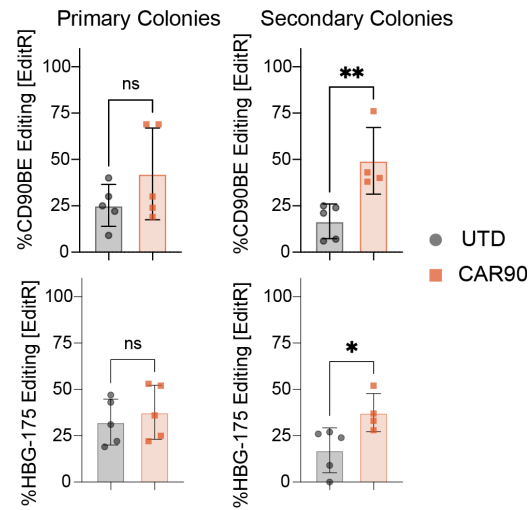


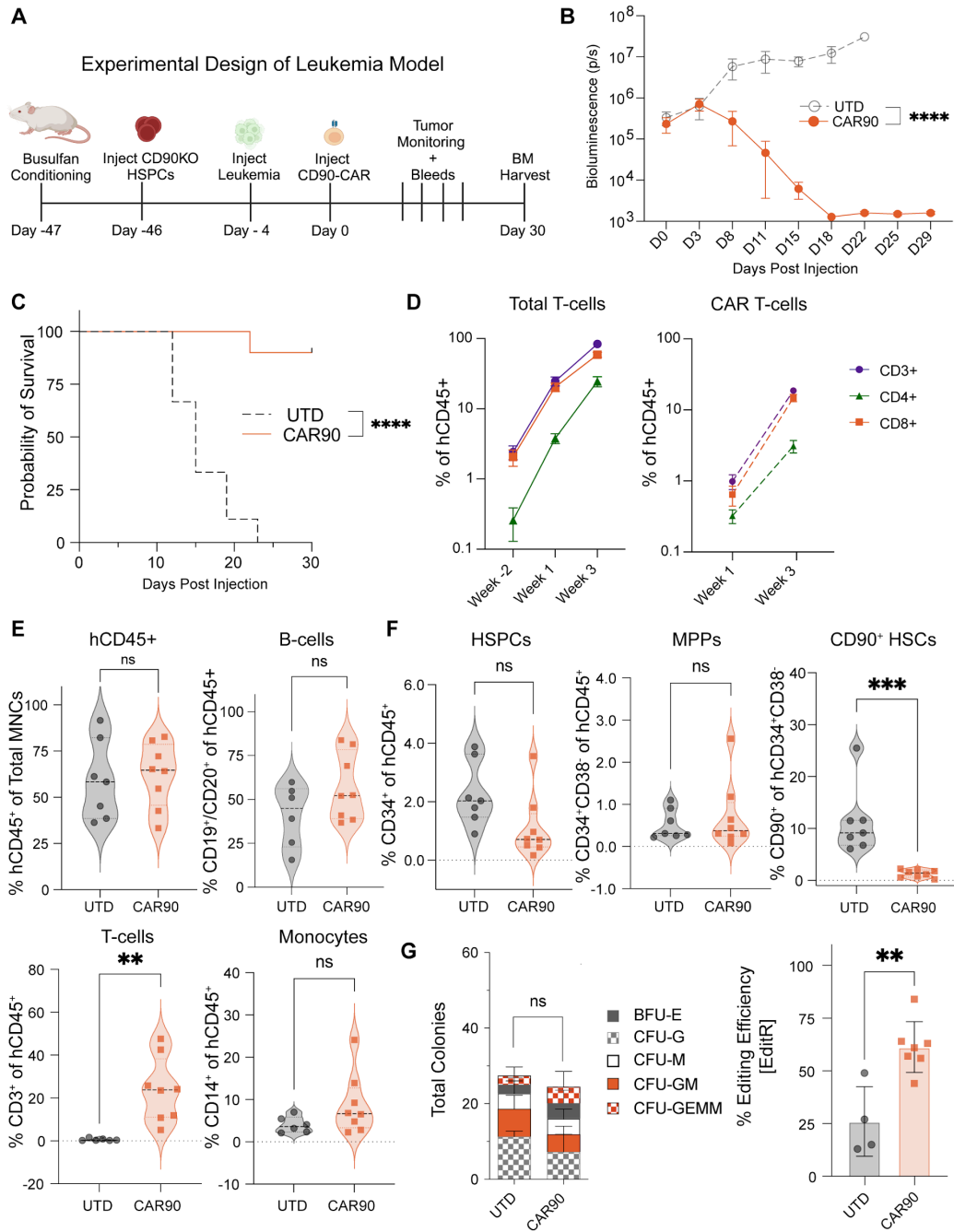








A**Experimental Design of *In Vivo* Enrichment Model****B****Day 7 Post T-cell Injection****C****D****E****F**



4.5 Methods

Study Design

This study aimed at evaluating the role of CD90 in adult and neonatal hematopoiesis. To investigate this, lentiviral shRNA constructs targeting CD90 were used to explore the impact of CD90 knockdown on HSPC engraftment potential as well as in vitro endothelial-to-hematopoietic transition. The impact of CD90 knockdown on in vitro EHT was further assessed by single cell RNA sequencing. We then developed a bona fide CD90 knockout system leveraging an ABE, demonstrating CD90KO HSPC engraftment potential in an NBSGW xenograft model. Next, we explored the impact of CD90KO in the clinically relevant NHP model. Concomitantly, we developed a CD90-directed CAR T cell and demonstrated their capacity for specific cytotoxicity, activation, and pro-inflammatory cytokine production. We then treated NBSGW mice engrafted with CD90KO HSPCs with CAR90 to investigate the safety of CAR90 in vivo. Finally, we employed CAR90 in two translationally relevant cancer models utilizing the CD90 overexpressing Jurkat cell line or primary patient AML samples. All experiments were conducted in at least duplicate, with the number of replicates and statistical analyses detailed in the corresponding figure legends.

Cell lines

K562 myelogenous leukemia cells (ATCC, X) were cultivated in RPMI-1640 (X) supplemented with 10% fetal bovine serum (FBS; source) and penicillin-streptomycin (Pen/Strep; 100U/mL; source). Jurkat T-cell leukemia cells (ATCC, X) were cultured in RPMI-1640 supplemented with 10% FBS and Penn/Strep (100U/mL). Human embryonic kidney 293T cells (ATCC, X) were cultivated in DMEM

Human CAR T cell production

Plasmids for viral vector production were acquired from the center for cellular excellence in hematology (CCEH) vector core. Our transfer plasmid contained a second-generation CAR backbone with a 41BB costimulatory domain and bicistronic truncated EGFR (tEGFR)

transduction reporter. α CD90 short chain variable fragments (scFvs) were designed based on the 5E10 antibody and ordered from GeneArt as plasmid DNA. α CD90 scFvs were introduced to our CAR vector by restriction enzyme digestion and ligation.

Third generation replication deficient lentivirus was acquired from the CCEH vector core for all in vivo and in vitro assays. Frozen human peripheral blood mononuclear cells (PBMCs) were acquired from the CCEH. T cells were isolated using the Human T cell Isolation Kit from StemSpan. Post-isolation T cell purity and CD4:CD8 ratio was assessed by flow cytometry. T cells were cultured in R10 at a density of $1 \times 10^6/\text{mL}$ and activated with CD3/CD28 Dynabeads at a 3:1 ratio. 24h after activation, T cells were transduced with CAR90 lentivirus at an MOI of 3. Media was doubled every other day and T cells were transferred to new culture vessels as appropriate. Dynabeads were removed on day 7 of culture and assessed by FACS for CD4:CD8 ratio and CAR positivity. T cells were allowed to continue expanding until day 10/11. T cells were then harvested for use in vitro and in vivo.

HSPC/CAR90 co-culture assays

HSPCs were acquired from the CCEH and thawed 24h prior to assay start. HSPCs were cultured in SFEM-II media with cytokine supplementation (FLT3 at X, TPO at X, and SCF at X) at a cellular density of $1 \times 10^6/\text{mL}$. 24h after thaw, HSPCs were split into treatment conditions at a density of $1 \times 10^6/\text{mL}$ and mixed with T cells at a ratio of 1:1. HSPCs were allowed to co-culture for 24, 48, or 72h with T cells, then had supernatant harvested for cytokine analysis and cells assessed by FACS for HSPC identity. CountBright counting beads were added to all samples to allow determination of total cell counts per manufacturer's instructions.

Concomitantly, HSPCs were sorted by FACS directly into tubes containing SFEM-II colony forming media, targeting 100 cells per mL. After sorting, our cell/media mixture was thoroughly mixed by vortexing, then plated into Xcm plates and cultured at 37C for 10 days. After primary

culture, total colony number and ID were assessed. Cultures were melted using warm PBS, then washed twice before replating 10% of total product into secondary colony forming assays. Secondary cultures were allowed to incubate for 10 days, and were analyzed as primary colonies were.

Flow cytometry

All flow cytometric analysis of cells was performed on BD FACSymphony A5. Cytokine bead array analysis was done with the BD LSRFortessa X-50. Cell sorting was carried out on the BD FACSymphony S6.

In vitro cytotoxicity assay

HEK293T cells were engineered to overexpress the CD90 protein with a bicistronic GFP reporter. 24h before assay start, HEK293T cells were plated into a flat bottom 96 well plate at a density of 10,000 cells per well in 100uL of complete media (DMEM + 10% FBS + 1% P/S). This assay was initiated by the addition of T cells at 10:1, 5:1, 2.5:1, or 1.25:1 effector to target (E:T) ratio in 100uL of R10. Plates were then imaged on the Incucyte microscope in the Fred Hutchison Shared Imaging Core hourly for 72h. Images were analyzed on the Incucyte analysis software for number of GFP+ cells at each timepoint and normalized to total GFP+ counts at 0h.

Cytokine bead array

Supernatant from HEK293T cell assays and HSPC assays were thawed from -80C on the day of cytokine bead array (CBA). Supernatants were assessed using the BD Th1/Th2/Th17 CBA kit per manufacturer's instructions. Stained samples were analyzed on a BD LSRFortessa cytometer. Standard curves were generated on Microsoft Excel and used to map fluorescent intensities to cytokine concentrations.

Conclusionary Remarks

This dissertation reports a cohesive body of work utilizing the globular glycoprotein CD90 as a therapeutic handle for genetically modifying, molecularly programming, and ablating HSCs. As such, the platforms discussed can be used to democratize HSC gene therapy in resource-limited countries. Future directions will involve utilizing each part of the platform to develop a cohesive protocol for the treatment of hematologic disorders. For instance, Cocal-derived CD90-MVP loaded with Cas9 and STAT5 can be injected into patients with sickle cell disease to reactivate fetal hemoglobin. To enable multiplexed enrichment and increase gene-modified chimerism, the transfer gene utilized can contain both an HBG promoter-targeted gRNA and a CD90 locus-targeted gRNA. As such, HSCs that have been therapeutic gene-modified to reactivate HbF will also lose CD90 expression. This future strategy could be used to enhance the efficacy of a second dose of CD90-MVP or, in conjunction with a CD90-targeted immunotherapy strategy, eliminate unmodified HSCs. Even still, before this strategy can be utilized further, scaling of the manufacturing and protocol must be completed in a nonhuman primate. This next step is likely to succeed due to other similar platforms that have succeeded using Cocal-based viral particles for in vivo gene therapy.

Another future direction of interest due to this body of work is the potential of in vivo programming using transcription factor-loaded MVPs. The capacity to target a specific subset and then deliver functional transcription factors enables the possibility of changing cell physiology in vivo without the removal of a cell type. While in this case, I have only evaluated the capacity to induce proliferation, the correct combination of transcription factors can also induce senescence, differentiation, and dedifferentiation. If true, and if the system can be expanded to other cell types, the platform described could have broad utility in the field of regenerative medicine.

Ultimately, further research is needed to gain a deeper understanding of the role of CD90 cellular physiology in the context of cancer stem cells. Increased CD90 expression correlates with a malignant chemoresistant cancer phenotype in hematologic and solid tumors[80]. While this dissertation begins to elucidate the role of CD90 in integrin biology and VEGF response, how this contributes to the production of a pro-survival phenotype was not fully elucidated. A more robust knockout of CD90 in cancer stem cells could be a valuable model for further elucidating the biology.

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